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The Editor comments

THE "NEW LOOK"

When you received this issue of THIS JOURNAL, we hope you noticed a number of significant changes in its appearance and make-up. These innovations, which were decided upon after much consideration, consultation, and debate, were adopted for several reasons. Certainly, it was hoped that the plastic surgery performed on the face of the publication would serve to improve further its appearance and esthetic qualities. An even more important aspect than the external plastic surgery, however, is the orthopedic surgery worked upon the body of the issue. In this regard the reader should particularly note the various new features which are being inaugurated. The scope of the publication has been expanded to permit the inclusion of critical review articles, editorials, articles of a technical nature, and reports of proposed drug assays and specifications.

With this extension in publication policy, no purpose appeared to be served by continued issuance of *Drug Standards* as a separate periodical. Hence, the latter publication has been merged into the present one.

Another change of special note is the retitling of THIS JOURNAL. The former name, *Journal of the American Pharmaceutical Association, Scientific Edition*, was criticized as being nondescriptive with regard to content, too unwieldy, easily subject to confusion with the *Practical Pharmacy Edition*, and difficult to cite correctly in literature references. The new title, *Journal of Pharmaceutical Sciences*, appears to overcome all of these objections. We are well aware that librarians and others responsible for the indexing and cataloging of periodicals will not receive this change with enthusiasm. We believe, however, that any inconveniences arising will rapidly disappear, and that from a long-range view, the change may be looked upon as beneficial even to them.

We are also aware that some readers will view with nostalgia the passing of the old title. We can only request that these individuals be tolerant, and that they, along with our other readers, accept the new features as an earnest attempt upon the part of the editors to improve the appearance of THIS JOURNAL, and to enhance its prestige, usefulness, and reader-interest.

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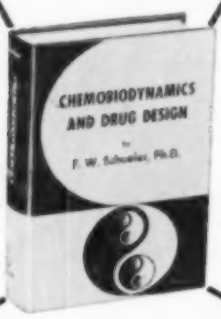

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by F. W. Schueler, Ph.D.

Professor and Chairman, Department of Pharmacology, Tulane University, School of Medicine, New Orleans, Louisiana.

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Form of Presentation.—Titles of manuscripts should be kept as short as possible and preferably restricted to one line. The name of the laboratory in which the work described was done should be placed in a footnote at the end of the first page. Each manuscript should begin with one or more introductory paragraphs indicating the intention and meaning of the investigation and the method of procedure. The introduction should be followed by a report of the essential details of the experimental work, and a discussion or interpretation where necessary. Authors may find it helpful to arrange manuscripts to conform to the style of presentation as illustrated in papers on analogous subjects published in recent issues of THIS JOURNAL.

In preparing manuscripts for publication in THIS JOURNAL, center headings should be avoided as far as possible. Side headings followed by a period and dash should be indented and run into the part of the text to which they apply. Such side headings may be used as freely as desired. Literature citations should be numbered consecutively in order of appearance in the manuscript and should be indicated by full-sized Arabic numerals enclosed in parentheses. If a literature reference is cited more than once in a manuscript, subsequent citations should be indicated by the first number assigned. References to footnotes throughout the text should be numbered consecutively by superscript Arabic numerals, but such references in each table should be designated by superscript lower case letters beginning with "a" and continuing in natural order. Literature citations should be grouped at the end of the manuscript under the heading "References." All bibliographic references should be checked with the original publications. The citations should be numbered consecutively with full-sized Arabic numerals enclosed in parentheses corresponding to the numbers used in the text. The names of all periodicals cited in the list of references must be abbreviated in accordance with abbreviations given by **Chemical Abstracts** in its "List of Periodicals Abstracted." The sequence followed in the citations must be as follows:

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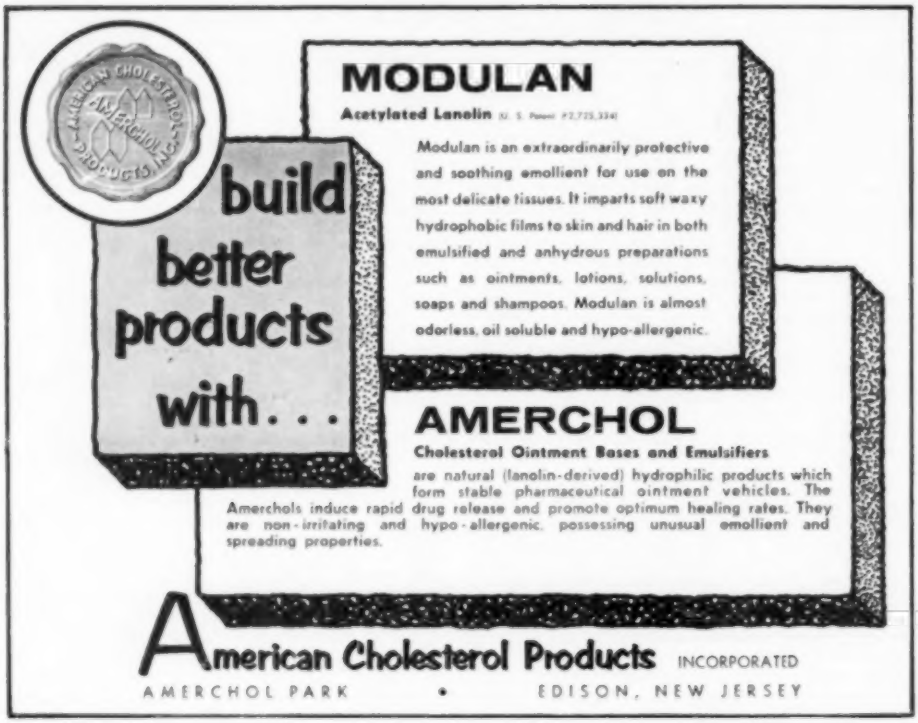
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NOTICE

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The next annual meeting of the American Pharmaceutical Association will be held in Chicago, Illinois, April 23–28, 1961. Titles and abstracts (not to exceed 200 words) of papers must be submitted to the appropriate Section secretary not later than February 1, 1961, or preferably earlier, in order to assure a place on any Section program. Papers should be sent to the appropriate Section secretary; the names and addresses of these secretaries are listed below.

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JOURNAL OF Pharmaceutical Sciences

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Review Article

Radioisotopes in the Pharmaceutical Sciences and Industry

By JOHN E. CHRISTIAN

HEVESY (1) recalls making numerous attempts to separate radium D from lead and he worked almost two years at this task without success. To make the most of this situation, Hevesy had the brilliant idea of using the radioactive radium D, now known to be lead^{210}, as an indicator of lead, thus profiting from the apparent inseparability of the two materials. This was the first instance of the utilization of radioisotopes as tracer substances and Hevesy, with the assistance of Paneth, reported (2) in 1913 the use of this tracer method for the determination of the solubility of some very slightly soluble lead compounds such as lead chromate.

Although the basis was laid, isotope utilization did not hold a prominent place in experimental procedures until the 1930's, due to the fact that the value of the technique was not recognized and radioactive isotopes were not readily available. However, with the availability of artificially-produced radioisotopes after the construction of the first cyclotron in 1932 (3), investigation of the use of isotopes increased. Artificially-produced radioisotopes were available only in very small quantities from accelerator machines and were quite expensive to produce. Consequently, it was not until after reactor-produced radioisotopes became available in quantity in the late 1940's that radioisotopes found extensive use.

Applications in the pharmaceutical sciences had their beginning quietly and unobtrusively in the Physics Laboratory at Purdue University where Lark-Horovitz and Leng reported in 1942 (4) a method of testing enteric coatings using sodium^{24} as a radioactive label. Although the method described would not be used indiscriminately in human beings today because of health hazard considerations and the regulations of the Atomic Energy Commission, the application was fundamentally sound and undoubtedly did much to stimulate thinking about potential applications in the pharmaceutical sciences. The same can be said for the work reported by Johnston and Lee (5) the following year which described a method of testing human skin absorption of sodium ions from different ointment bases using sodium^{24}.

The pharmaceutical sciences still did not awaken to the unique opportunities afforded by the use of radioisotopes as tracers and sources of ionizing radiation until articles (6–10) specifically pointing up potential applications and advantages were published in 1947 and 1948. At that time there was no instruction in this or other areas being provided by either pharmacy graduate departments or by other scientific disciplines for that matter; and furthermore, there were no

Received from the Bionucleonics Department, School of Pharmacy, Purdue University, Lafayette, Ind.

pharmaceutical manufacturers utilizing radioisotope techniques in their research. In the past twelve years, however, there has been an acute awareness of the potentialities and value of radioisotope utilization. This is evidenced by the great increase in scientific publications describing data obtained with radioisotopic methods, the number of pharmaceutical manufacturers engaged in the use of radioisotopes, and the number of pharmacy schools teaching isotope technology. According to a recent survey (11) 32 pharmaceutical companies stated they were engaged in the use of radioisotopes at the end of 1959 and 17 additional companies stated that they planned to initiate a radioisotope program by 1961. This represents 45 per cent of the companies which supply in excess of 90 per cent of the pharmaceutical and medical products in the United States. A total of 418 scientists, of whom 139 (33%) are on a full-time basis and 279 (67%) are on a part-time basis, work with radioisotopes. Over 40 per cent of these people have received formal training in radioisotopic tracer and radiation techniques. In addition, the survey shows that over 50 companies have contracted for radioisotope and radiation research to be carried out by outside organizations. Perhaps more striking is the fact that 18 schools of pharmacy offer specific courses in radioisotope technology and an additional 23 schools plan to initiate such courses by 1961. A total of over 1,780 students have been trained to date in these instructional programs. Thus, the growth in training programs and research applications during the past 12 years has been phenomenal and most certainly reflects the considered importance of radioisotope and nuclear techniques in the pharmaceutical sciences.

PRINCIPLES OF APPLICATION

The principles of application of radioisotopes have been adequately described in a number of publications (7, 10–13) and need not be elaborated extensively here. However, it is important to reiterate the reasons why radioisotopes are continuing to find such an important place in pharmaceutical research as tracers and as sources of ionizing radiation for a whole host of potential yet untried applications in solving many pharmaceutical research and production problems. All applications of radioisotopes, whether for research, analytical, or production purposes, are based on three simple principles. The first of these is that radiation has a definite and measurable (if a sufficient quantity of radiation is used) effect on all matter which it strikes or penetrates. This principle may be utilized in radiation sterilization, pasteurization, light production, induction of chemical reactions, production of biological mutations, destruction of living cells (therapeutic application), dissipation of static charges, and in bringing about changes in physical properties, such as specific gravity, surface tension, and viscosity.

The second principle is the reverse of the first, that is, matter affects radiation. This is manifested by radiation absorption, reflection, and scattering. The measurement of thickness, density, liquid level, moisture, and the procedures of well-logging, radiography, and autoradiography, as examples, are all based on this principle.

The third principle involves the use of radioactive atoms, molecules, or compounds as tracer substances, making it possible to follow these materials (with the ultimate in sensitivity, specificity, and attention to complexity of details) through complex chemical, biological, or technical processes. As tracer substances, radioactive isotope applications are unlimited in scope and the principle is employed in studies of the metabolism of trace constituents in plants and animals (including man), studies of the elucidation of chemical reaction mechanisms, studies of gas, liquid, and solid flow, determination of the thoroughness of mixing and separation, and general use as industrial tracers. This principle is also applied in the use of radioactive isotopes as diagnostic agents in disease and malfunction conditions of various description.

In the case of many radioisotopes, another important factor contributing to their versatility as tracer substances is the ability to detect the radiation emitted at a distance and through varying thickness of materials, providing for remote and accurate detection and analysis by a completely nondestructive technique. This is important as regards human diagnostic tests and also makes radioisotopes extremely valuable in automatic industrial processes of various description.

Fortunately, usable radioactive isotopes are available for most of the 102 elements in the periodic table. Exceptions to this include the following elements: helium, lithium, boron, neon, nitrogen, and oxygen; with all the other elements, usable radioisotopes are presently available. Of the 102 elements in the periodic table, over 900 radioactive isotopes have been characterized to date.

The advantages and the versatility of radioisotopes as tools in medical and pharmaceutical research and industrial applications provide for a

broadening of utilization which is limited only by our ingenuity in devising new areas of application.

APPLICATIONS IN PRACTICE

Determination of Release, Absorption, Penetration, and Permeability.—From a strictly pharmaceutical viewpoint radioisotopes have been applied in this category more than in any other. This is probably true because researchers have long been searching for ways to determine the amount and rate of absorption of medicaments and other substances from various formulations and preparations. In addition, it is desirable to be able to study the permeability and penetration of medicaments through the skin and other living membranes. The effect of the physiologically inert formulation ingredients included in various dosage forms, and the physical-chemical properties of the active ingredients as a function of its release, absorption, penetration, permeability, and retention are of utmost importance in the development of effective forms of medication. Such studies, more often than not, demand the detection and measurement of minute quantities of the active ingredient in complex biological materials over extended periods of time. Radioisotopes, therefore, are a natural tool for such studies. Gemmell and Morrison (14), in a review article discussing the release of medicinal substances from topical applications and their passage through the skin, state that isotope techniques offer the most accurate *in vivo* means of assessment; they are of particular value when the biochemical processes following absorption are being investigated.

As previously referred to, the first pharmaceutical applications involved the determination of medicament release time from enteric coatings (4) and the release and skin penetration of medicament from ointment bases (5). Cyr, *et al.* (15), described the results of a study of the comparative absorption of sodium iodide (I^{131}) from lard, wool fat, and petrolatum. The extent of absorption was measured by determining the amount of I^{131} in the thyroid gland after sacrifice of the animal. This is an extremely sensitive method of detection of absorption of iodide since the thyroid normally concentrates iodine up to 10,000 times the amount found in other body organs, including blood. The big advantage, in addition to being able to detect the minute quantities of iodide ion penetrating the skin, is the ability, through the isotopic tracer method, of selectively and accurately making these measurements in the presence of iodine normally in the body, and that subsequently taken into the body through food and water. No other analytical technique presently available offers this advantage. The determination of the radioactivity of the thyroid gland, as a measure of absorption through the skin, is limited to iodine studies and the results are subject to variation due to differences in thyroid activity from animal to animal. Thus, a number of animals must be studied in order to arrive at statistically significant results and the method is not generally applicable to a variety of other materials which one might wish to study. The results obtained by this and similar procedures are only relative and provide little information about the total material absorbed. Skauen and co-workers (16) used the above described procedure to study the absorption of iodide ion from 37 different ointment bases of varying composition and the authors were able to draw specific conclusions about skin penetration from different formulations. The authors were careful to point out, however, that in order to determine more accurately the relative efficiency of some bases, the method employed would require modification. Plein and Plein (17) used the above method to determine the absorption of sodium radioiodide (I^{131}) from six different ointment bases. The radioactivity of the iodine^{131} ointment-treated skin was determined as a measure of the penetration of the sodium iodide.

Clinical studies involving these basic principles were carried out quite early on human subjects using the naturally-occurring radioactive isotope of radon. Lange and Evans (18) applied an ointment consisting of radon in lanolin to the skin and measured the amount of radioactivity in the expired air as a measure of the penetration into the blood stream. Thorium X was applied to human skin to demonstrate that penetration is by way of the appendages (19), confirming work by others (20, 21) who studied biopsies of tissue which had been treated with thorium X in various vehicles.

Although evaluation of ointment bases was not involved, Loeffler and Thomas (22) determined the absorption of strontium through intact and broken rat skin using radioactive strontium. Severan and Tack (23), using radioactive zinc in the form of zinc sulfate, showed that absorption was least from fat bases and could be increased by the addition of soap. The absorption of topically applied methionine labeled with S^{35} to guinea pigs has been evaluated by measuring the presence of S^{35} in the cystine in newly grown hair (24). Hadgraft, *et al.* (25), took the approach of

studying percutaneous absorption by measuring the radioactivity of blood samples. They chose diiodofluorescein (I^{131}) because it was readily available and possessed solubility characteristics similar to those of a number of substances commonly applied to the skin. The measurements of radioactivity were made on 10-ml. blood samples and the quantities of iodine^{131} found in the total blood sample was quite low. The procedure gives no indication of the total label absorbed since the compound being studied may be removed from the blood stream almost as soon as it is absorbed, and perhaps returned to the blood stream in variable amounts at variable time intervals later with absorption, concentration, and excretion taking place all at the same time. Other analytical methods based on blood concentration also suffer from this disadvantage. This probably explains the extremely small amount reported as absorbed and the wide animal variations. The results are probably not very meaningful since they are based on averages of only three animals. The authors state that to obtain results suitable for statistical analysis large numbers of rats would have to be used, raising difficulties of added costs, radiation hazards, and disposal of contaminated animals. The proper approach would not be to use more animals but to develop a method of analysis which would provide less variable and more meaningful results. Radiation hazards are of little consequence in this type of experiment; certainly no more hazardous than many other materials used in the research laboratory. Disposal of iodine^{131}-contaminated animals, even in large numbers, is also of little consequence at the level employed here since iodine^{131} has only an eight-day half life. Thus, the fault of seeming failure of this method to yield reliable results is not in the isotopic method but in the proper design of the experiment to yield valid data. However, it is through this type of experiment, with initial limitations because of one factor or another, that more accurate and precise methods based on increased knowledge and better instrumentation can be made available.

Katz and Barr (26) reported their studies of the rates of absorption of iodide ion from tablets prepared for sublingual absorption. Iodine^{131} was used as the label in the form of sodium iodide incorporated in the tablets which were administered sublingually to anesthetized mice. The relative iodide uptake was determined by measuring the radioactivity of the blood flowing through the tail of the animals. The tail was placed in a specially shielded detector for the measurement. As the authors have clearly pointed out, determining the level of radioactivity through the tail is a much simpler and more convenient method than chemical analysis of blood samples and/or sacrificing animals and removing organs at periodic intervals. However, sensitivity is sacrificed since the circulating blood in the tail of the animal represents only a small proportion of the total blood.

Sheinaus, *et al.* (27), showed the effect of topically applied hyaluronidase on cutaneous permeability to iodide ion. The only possible approach to this type of investigation was through the use of iodine^{131} because of the minute quantities of iodine present in the 50-mμ thick skin sections taken for permeability evaluation. This method is applicable to any labeled compound one might wish to study. Ointments containing iodine^{131} in the form of iodide ion as the controls were applied to the skin of guinea pigs immediately adjacent to the test ointments which were identical to the controls except for the addition of 750 turbidity-reducing units of hyaluronidase. After elapse of the desired experimental period, the properly prepared circular skin punches were sectioned into approximately 5-μ thick samples and the total radioactivity of each section was determined. In this way, the depth of permeation, the total permeability, and the effect of hyaluronidase were determined in different ointment base formulations. In conjunction with autoradiographs of the skin sections (although this was not reported by the authors) this method offers an almost ideal approach to studies of permeability and routes of permeation and penetration. This method, however, would give no information on the quantity of penetration into the blood stream.

Sorby and Plein (28) describe a radiometric method for determination of the absorption of mercury from ammoniated mercury ointment bases through the intact and abraded skin of rats. Ammoniated mercury (Hg^{203}) was used. Twenty-four hours after application of the ointments, both kidneys were removed and assayed for their radioactive mercury content. Again this type of evaluation is limited to a specific material and provides only relative data as to absorption through the skin. Even with the limitations cited, however, these methods are superior to others that have been used because of the sensitivity of detection and specificity.

Radioisotopes have likewise been used to advantage in *in vitro* tests of one type or another. Stark and his colleagues (29) describe an *in vitro*

method designed to study medicament release from pharmaceutical semisolids. Radio-labeled mercuric and iodide ions were incorporated into hydrophilic bases, emulsified with anionic, cationic, and nonionic surface-active agents, and the release determined through a cellulose membrane. The membrane was attached to a chamber containing a circulating physiological solution, the radioactivity of which could be continuously determined as a measure of the medicament release. The device provides a certain elegance of technique but suffers from the usual difficulty of the arbitrary value of most *in vitro* methods.

Redman, *et al.* (30), using a modification of the device described by Stark, investigated the effect of different suspending agents in two different concentrations on the dialysis of the iodide ion labeled with iodine^{131} through a semipermeable membrane.

Madison and Christian (31) reported studies on the absorption of sodium ion in the rat after introduction of sodium^{22} or sodium^{24} into the gastrointestinal tract and after subcutaneous injection. The extent of absorption was determined by measuring the radioisotopic content of blood samples taken at periodic intervals. The effects of various osmotic pressures, various pH's, and certain astringent agents on normal absorption were determined. A similar type of study was reported by Lormand and co-workers (32) in which the effect of hyaluronidase on absorption using sodium^{24} as the tracer was used.

Using the basic idea reported by Lark-Horovitz and Leng(4) in their study of enteric coatings in humans, Peterson and co-workers (33) described a procedure for testing enteric coatings in rats. The method involves coating small pills containing sodium^{24}, administering them to a rat, and determining the time of disintegration by the appearance of radiosodium in the circulation. The exact site of disintegration was determined by sacrificing the animal and observing the location of the gentian violet color which had been included in the constituents of the pill to make location of the pill remains easy. The results were compared to the results of an *in vitro* method. Peterson and his co-workers (34) also studied suppository bases using iodine^{131} to compare the rate of absorption from rectal suppositories administered to rats. Five separate bases were studied by the incorporation of 3 mcg. of sodium iodide labeled with iodine^{131}. At various time intervals after administration, the treated animals were sacrificed, the blood, thyroid glands, and colon containing the unabsorbed portion of the suppository were collected, and radioactivity of aliquots of each were determined. In this manner, a relative comparison of the absorption of iodide ion from the various bases was obtained. Riegelman and Crowell (35–37) published a series of three papers using essentially the same procedure as described by Peterson with the exception that the iodine^{131} measurements were made externally and continuously. A thallium-activated sodium iodide crystal placed in a 1-inch shielded collimator was used as the detector. The rat was placed over the collimator in the desired geometrical position, as determined in preliminary studies, to result in maximal counting from the rectal area in which the rectal dosage was placed. Thus the measurement was of the percentage of dose remaining in the area and the assumption was made that the difference from the original activity represented what was absorbed. Studies were made on the absorption of sodium iodide (I^{131}), iodoform (I^{131}), and the sodium salt of 2,4,6-triiodophenol (I^{131}) in various formulations. One experiment was carried out by determining simulated continuous blood levels by counting the head portion of the animal. It is apparent that a number of factors must be carefully controlled to give satisfactory results from the method; the geometric placement of the dose and its retention within a narrow and specific section of the intestine, the nature of the vehicles to be sure that none of the material leaked beyond the plugs used, the degree of anesthesia required to maintain the animal in a specified position over the collimator may in itself effect absorption, and the maintenance of exact geometrical placement over the collimator. Although the collimator was stated to restrict the detection of activity to approximately 4 cm. in length of the rectal canal, activity from a portion of the absorbed dose would also be detected and probably would vary significantly, depending on the placement of the animal on the collimator. All of these factors most certainly would be difficult to control under the best of conditions and would provide probability of considerable error. More serious than these factors, however, is the determination of absorption by difference between the original and remaining activity. A very slight loss or displacement of the original dose (high in activity) would result in a gross error in the calculation of the absorbed dose. The results presented are based on a bare minimum number of animals, four in each case.

Rather extensive studies of the iodide ion permeability of frog membrane and the effect of astringent substances on such permeability have been made using iodine^{131} as a tracer (38–41).

Davson and Danielli (42) as early as 1943 had suggested that radioactive tracers should be extremely useful in such studies. Earlier studies using other methods were often unreliable. The use of iodine^{131}, however, has clearly indicated that radioactive tracer studies provide for a precise and rapid method and permit the study of permeability in the presence of foreign substances. In addition, this procedure permits the study of the route of penetration using the autoradiographic technique (39). On studying the effect of astringent materials on iodide ion penetration, it was found that these materials increased permeability and that the amount of increase is proportional to the concentration. The procedure was developed and applied for the evaluation of antiperspirant properties. Nelson (43) reports the use of the above method for routine evaluation of antiperspirant properties and states that no other satisfactory method which will effectively evaluate astringents with respect to antiperspirant properties is presently available to the cosmetic industry. He points out that with slight modifications this technique has been used to study other classes of compounds. With the use of tritiated water, for example, the effect of such materials as "barrier creams" may be evaluated as to their properties of preventing penetration of water through a skin membrane.

Thoms, *et al.* (44), as early as 1948 described the use of radioactive tracers for the determination of residues on the skin. This application of radioisotopes is of outstanding significance in this field of study and stands today as the best method available with no apparent disadvantages. Sensitivity of detection is of prime importance here and other investigators have found the method applicable to solving their problems. Studies have been made on the retention of sodium iodide, iodoform, and dimethyl phthalate in various emulsified ointment bases, including o/w and w/o types (45). *In vivo* studies in white rats have been made to determine the length and amount of tooth decay inhibitors in the oral tract (46, 47) and these same references refer to the use of the method in skin retention studies involving bacteriostatic agents, soap formulations, and emollients from a lotion formulation. Nelson (43) refers to the use of similar procedures for detergent evaluation in removing soil from various surfaces, textile fibers, hair, and membranes (including living skin), for studying absorption onto human hair, and for studying retention on human teeth. In the latter category various agents retained on the teeth from dentifrice formulas and solutions have been studied. Among the tracers which have been used here are calcium^{45}, iodine^{131}, zinc^{65}, phosphorus^{32}, fluorine^{18}, sulfur^{35}, rubidium^{86}, potassium^{42}, sodium^{24}, and carbon^{14}-labeled compounds of various description. Jarvis, *et al.* (48), and later Urakami and his associates (49) used isotope tracer techniques to study phosphorus and calcium depletion of teeth after exposure to various solutions.

Barker and co-workers (50) describe the technique and results obtained when several hydrophilic ointment bases were tested by a modified agar plate method using radioactive tracer techniques for determining the zone of penetration in the agar.

Discussion in this area has been emphasized and been made reasonably complete since the problems involved here are more pharmaceutical in the strict sense of the word. A consideration of the use of radioisotopes in solving the problems referred to reveals a certain elegance of technique and, in several instances, the use of radioisotopes leaves little to be desired as a means of evaluation. Nevertheless, fundamental bases are still lacking upon which experimental work, to be of practical value, can be placed for a large share of the problems encountered in this area. *In vitro* methods are at a great disadvantage to begin with, regardless of the technique of measurement, and *in vivo* studies in various animal species yield various results, in themselves difficult to extrapolate into human evaluation. This is not stated to be unduly critical of the experimental methods to date because experimental approaches described, with their initial limitations of one factor or another, provide the groundwork for more accurate, precise, and meaningful methods as information, knowledge, and instrumentation advance. Undoubtedly many of the experimental procedures described using radioisotopic methods will lead to more refined techniques in human subjects resulting in the practical evaluation of medicament forms under exact conditions of intended applications. With new whole body liquid scintillation detectors, permitting the measurement of natural levels of radioactivity in the human body, presently being constructed, and other specialized equipment on the design boards, the determination of release, absorption, penetration, and permeation, and other qualities of various medicament forms in human subjects will be perfected. As an excellent example of the usefulness of radioisotopic tracer techniques in pharmaceutical product evaluation, sustained-release type of medication

is currently being evaluated in human subjects under precise conditions of intended usage. The importance of this type of evaluation cannot be overestimated as we move into the use of more elaborate medicament forms designed to produce a specific and carefully controlled effect.

Drug Metabolism.—The development of medicinal products and their safe and intelligent use requires fundamental studies in biochemistry, biology, pharmacology, microbiology, endocrinology, and related areas. These branches of biological science have made extensive use of radioisotopes as tracer substances and the pharmaceutical industry has made numerous applications of the techniques; so numerous, in fact, that it is not practical to provide more than a brief discussion in this paper of what has been accomplished. The radioisotope technique makes possible with relative ease a study of the rate of absorption, excretion, blood concentration, distribution in various organs, and residue properties of almost every medicinal substance and the accompanying ingredients in the formulation. More important still is the scientific approach to fundamental drug mechanism studies provided by radioisotopic techniques. This use of labeled drugs in pharmaceutical research has become commonplace and only a few references will be cited here as examples of what has been and can be done. Fingl, *et al.* (51), reported some of the early work in this field in describing the tissue distribution of sulfur^{35} in the rat following the oral administration of labeled sulfanilamide. Taylor and co-workers (52) reported later on the metabolism of sulfur^{35} thiopental. Other examples of this type of study include experimental data on cobalt^{60}-labeled vitamin B_{12} (53), carbon^{14}-labeled meperidine (54), carbon^{14}-labeled phenurone (55), carbon^{14}-labeled reserpine (56–59), iodine^{131}-labeled insulin (60), carbon^{14}-labeled acetylsalicylic acid (61, 62), sulfur^{35}-labeled chlorpromazine (63), N-methyl-C^{14}- erythromycin (64, 65), C^{14}-carboxyl salicylamide (66, 67), C^{14}-labeled acetylsalicylic, salicylic, and gentisic acids (68), iodine^{131}-labeled iodopropylidene glycerol (69), chlorpromazine-(N-methyl) C^{14} (70), reserpine H^3 (71), C^{14}-hexamethylenimine (72), Co^{58} cobalt chloride and certain cobalt-containing hemopoietic agents (73, 74), hydrochlorothiazide H^3 (75), promazine S^{35} (76), amphetamine C^{14} (77), and C^{14}-labeled hemicholinium No. 3. These are but a few examples of the labeled medicinal substances which have been studied in living systems. The advantages of the isotopic tracer method to this type of application are obvious, and when one considers that other methods are not available to give the desired answers, the inconveniences of using radioisotopes that are sometimes referred to become negligible.

Plant Biogenesis and Biochemistry.—The basic principles described for the use of radioisotopes in drug metabolism in animals hold equally well for plant work. The rate of uptake, distribution, metabolism, and elimination of various elements, radicals, and compounds in medicinal and other plants can be readily and easily studied with radioisotopes. Radioisotopes offer about the only approach to studies of plant biogenesis which are leading to fundamental information about living processes in general and to mechanism of drug synthesis in particular. Geiling, *et al.* (78), have shown the utilization of carbon from carbon^{14}-labeled carbon dioxide in the production of the digitalis glycoside, digitoxin. Youngken and Djao (79, 80) later studied the biogenesis of the digitalis glycosides from labeled acetate feedings using hydroponic culture methods and found the incorporation of the C^{14} to be significantly small. Other biogenesis work reported by Lieberman and co-workers (81, 82) involved studies of glycolic acid and acetate metabolism in *Bryophyllum calycinum* Salisb. In both instances, C^{14}-labeled compounds were infiltrated into the plant parts and the metabolic products separated and identified by paper partition chromatography.

The plant biochemist has also been responsible for the preparation of labeled drugs by biosynthesis when labeling either is difficult or impossible by chemical methods. Examples of medicinal agents which have been uniformly labeled with C^{14} by biosynthesis include digitoxin and nicotine previously mentioned (78), veratrum alkaloids (83), colchicine (84), morphine (85), reserpine (86), and the ergot alkaloids (87, 88). Although the studies in plant biogenesis and biochemistry as conducted in the pharmaceutical sciences have been small in number in comparison to animal studies because of the declining importance of crude drug production, these studies are of great importance to fundamental studies of living processes, and plant scientists in general are using radioisotope techniques extensively to elucidate the many complex biochemical reactions involved.

Synthesis of Labeled Compounds.—One of the largest deterrents to the use of radioisotopically-labeled medicinals in all phases of pharmaceutical research, as well as other areas, is the difficulty of synthesis of many of the de-

sirable compounds with appropriate labeling isotopes in desired positions in the molecule. Thus, this area of research has been an important one and the pharmaceutical and medicinal chemists have been called upon to synthesize chemically the desired labeled compounds where possible. If chemical synthesis is not practical or possible, biological synthesis, as referred to above, is often used. Examples of published reports on the chemical synthesis of labeled medicinals include the synthesis of C^{14} vitamin B_{12} (89), Co^{60} vitamin B_{12} (90), C^{14} chloroform (91), S^{35} sulfa drugs (92), C^{14} carboxyl dimethyl phthalate (93), C^{14} carboxyl acetylsalicylic acid (94), I^{131} iodoform (95), I^{131} iodine monochloride (96), Au^{198} gold thioglucose (97), C^{14} carboxyl salicylic acid (98), I^{131} Congo red (99), and H^3 halothane (100). These are but a few examples; there are a multitude of others and new methods plus improvement of existing ones are continually being reported to provide high specific activity material in chemically and radiochemically pure state ready for use in tracer experimentation. It is now almost inevitable that each new outstanding drug developed will be synthesized with an appropriate label for use in obtaining, rapidly and easily, the multitude of data required prior to and during product formulation and human usage.

Pharmaceutical Analysis.—The broadest, overall application of radioactive isotopes to any area is in the field of analytical chemistry, since almost all research procedures (chemical, medical, pharmaceutical, biological, and others) begin and end with some type of qualitative or quantitative measurement. It is difficult to overemphasize the future analytical importance of radioisotopes in the pharmaceutical sciences. One of the biggest problems facing the pharmaceutical scientist today is the rapid and accurate analysis of minute amounts of materials in complex mixtures. More and more medicaments active in small quantities are being developed and these are being administered in complex pharmaceutical mixtures. Analyses of these mixtures are difficult. Furthermore, it is often desirable to determine minute quantities in biological samples after administration to man and animals. Radioactive isotopes as analytical tools are being used to solve some of the problems and the analytical uses to date are pointing the way to unlimited applications in the future.

A number of review articles on analytical applications of radioisotopes have been published (11, 13, 101–109) making it unnecessary to go into detail about the principles involved. However, a few selected papers will be cited here for purposes of discussion of the methods to point up their applications. Examples, to cite a few, of medicinal analyses using radioisotopic methods include vitamins (110, 111), steroids (112, 113), antibiotics (114–116), insecticides (117–119), barbiturates (120), the sulfa drugs (121), intrinsic factor (122, 123), and salicylic acid (124, 125). The most excellent example of the use and acceptance of the radioisotopic method is in the analysis of vitamin B_{12} (cyanocobalamin). The method described by Bacher, *et al.* (126), involving organic solvent extraction, ion exchange, and the use of a radioactive vitamin B_{12}-tracer has met all the tests of an excellent analytical procedure. With vitamin B_{12} cobalt^{60} available as a tracer, it is possible to assay complicated media for cyanocobalamin or total cobalamins and represents the most discriminating analysis available to date for these materials. The method has made the differentiation of cobalamins and biologically inactive cobalamin-like substances such as "red pigments" and pseudo vitamin B_{12} a practical one. Chaiet, *et al.* (127), have shown that pseudovitamin B_{12} does not give a response in this method. A collaborative study of the method by a joint U. S. P.-N. F. panel (128) clearly indicated the usefulness of the method and it was made official, replacing the microbiological methods in the U. S. P. and the N. F. in the fall of 1956. Prior to the incorporation of radioactive pharmaceuticals as official drugs in the U. S. P., sodium radioiodide (I^{131}) solution being the first, it was necessary to work out details of the monograph (129) including all the tests for contamination, radiochemical purity, chemical purity, and the assay procedure. The details of the development of the monograph have been published (130, 131) and represent another example of the application of radioisotopes to pharmaceutical analyses.

The problem of determining the stability of a new drug or dosage form has long been vexing to the drug manufacturer. The use of radioactive isotopes as analytical tools suggests itself as a method of studying stability and shelf life since it offers sensitivity and ease of detection, even in complex mixtures. Some ideas and procedures along this line have been reported (132–134); however, the details of general applications have yet to be developed.

A recent and novel method of analysis which should find many applications in the pharmaceutical sciences in the future is activation analysis. Alt'ough the method has been used

now for several years in other fields (135–138), there is only one reference to its use in the pharmaceutical sciences. Kaiser, *et al.* (139), reported the investigation of the slow neutron-activation analysis of arsenic and antimony-containing pharmaceuticals. The method was applied successfully to the analyses of arsenic trioxide, carbarsone, oxophenarsine, antimony potassium tartrate, and stibophen without sample processing and chemical separations.

It is reasonably obvious that radioisotope tracer analysis is particularly useful when the substance analyzed cannot be quantitatively separated from a mixture, when minute quantities are involved, or when the analytical problem cannot be solved by other methods. The procedures are not considered to be simple in application, since they require specialized skills by the analyst and may be quite complex and tedious until details are worked out. This, however, is not unique to radioisotopic methods since in the early stages of any new method, essentially the same difficulties arise. The success of the method is determined by the type of application and the thoroughness of operation. Like all other analytical procedures, the radioisotopic method cannot be used indiscriminately for solving a variety of analytical problems. However, the value of these methods where utilization is possible and justified cannot be denied, and we are certain to find radioisotopes being used as routine analytical tools in most pharmaceutical research and industrial laboratories in the near future.

Applied Industrial Uses.—Most of the major industries in America have realized by this time the great potential benefits of applications of radioisotopes to technical operations, process monitoring, and quality control, and have reported substantial benefits in quality of products as well as savings (12, 140–143). The use of radioisotopes as an applied industrial tool in the pharmaceutical industry, however, has not progressed at all, and apparently very little thought or effort has gone into this application except for the very limited effort which has been expended on the sterilization of materials. Controulis, *et al.* (144), exposed a number of pharmaceuticals in finished product form to irradiation by gamma rays from a Co^{60} source. Sterilization of purposely contaminated samples of the pharmaceuticals was effected successfully in all of the products studied. The authors state that except for glass discoloration and the possible deterioration of a limited number of products, such as pituitary substances, the sterilization of pharmaceuticals in finished package form via gamma radiation is considered to be a practical and valuable addition to the various sterilization methods used by the pharmaceutical industry. Perhaps these authors were somewhat optimistic since the industry has not, as yet at least, used the process on an industrial scale. The reason the potential of this method has not been fully realized is probably due to conflicting reports regarding possible detrimental effects of the radiation, fear that radioactivity may be induced in the product (an unfounded fear), concern over the position to be taken by the Food and Drug Administration, and the economic aspects of this method in competition with other methods.

In order for radiation processing techniques to realize their full potential, a systematic program of study and scientific evaluation of the effects of radiation upon specific chemical and biochemical entities should be undertaken. Studies of this nature have been carried out (145) but on a relatively restricted scale. An indication of the practicality of sterilization using radiation-producing machines on certain types of pharmaceutical products is demonstrated by the routine radiation sterilization of the surgical suture (146, 147).

The other known applied industrial uses in the pharmaceutical industry (11) are the isolated uses of (*a*) a beta-gauge on a calendaring machine by a bandage manufacturer, (*b*) a liquid level-gauge on a mixing tank, (*c*) a static elimination device, and (*d*) the limited use of electron sterilization on plastic bags. It would thus appear that the pharmaceutical industry has not taken advantage of the possibilities of radioisotope utilization in production problems and technical operations, even though the potential applications are many-fold and are readily apparent to those who give consideration to the possibilities. This whole area of application to the pharmaceutical industry is rather completely covered from all aspects in a report (11) covering the findings of a year's study of the industry. Numerous potential applications and examples are described.

Miscellaneous.—There are a few miscellaneous applications of radioactive isotopes reported in the pharmaceutical literature which do not seem to fit readily into any of the previous categories. Charipper and associates (148) report on the use of ionic radiation in the development of a bioassay for granulocytopenia. Leucopenic properties of substances administered to X-irradiated animals was demonstrated by a retarded rise in the blood count as compared to X-irradiated animals as

controls. Thus, the leucopenic properties of various substances can be evaluated in a more positive manner than by other commonly used methods. Numerof, *et al.* (149), discuss the use of phosphorus^{32} for the determination of vitamin D potency by measuring the deposition in bones of intact rachitic rats using a radiometric analysis. This method appears to exceed the range of the familiar "line test," is more rapid, and provides for a more precise interpretation. Beal and Skauen (150) employed the tracer technique to evaluate different procedures of washing hypodermic syringes. Blood was labeled with iodine^{131}, used to contaminate syringes, and then the efficiency of removal by different methods was evaluated by counting the residual radioactivity remaining on the syringe. Kopplin and co-workers (151), in studies on the adsorption of odorous materials on solid and liquid adsorbing surfaces, used C^{14}-labeled ethyl alcohol to measure the actual amount of alcohol adsorbed which was in turn responsible for a given change in surface potential. Surface-potential changes were being investigated as a possible means of measuring odorous materials. Since the amounts of material being measured on the surface were so extremely small, radioisotopic methods were considered to be about the only feasible method which could be applied. Banker, *et al.* (152), have applied radioactive tracer techniques to the study of the uniformity of distribution of several additive phosphorus^{32}-labeled compounds in tablet matrixes. Certain physical properties of the additive and excipient powders, the duration of mixing, methods of granulation and drying, solubility of the additive in the granulating agent, and additive migration were studied for their effect on the uniformity of additive distribution. The tracer method appears to be a rather ideal procedure for studies of this nature.

Medical Uses.—At least two pharmaceutical manufacturers are processing radioactive isotopes for use in medical diagnosis and therapy; however, a discussion of the medical application, as far as use in clinical practice is concerned, is outside the scope of this paper. For those who may wish specific information on these applications, a few references are cited which will initiate the reader into this important subject (153–155).

Potential Applications.—The previous discussion of the applications in practice of radioactive isotopes in the pharmaceutical sciences and industry illustrates the great versatility of use which is possible. It is reasonably obvious that the potential uses to which radioisotopes might be put in the future are myriad, ranging from unlimited tracer applications in the biological sciences, to gauging procedures with microcurie quantities, and industrial uses of high level radiation sources at the multicurie level. The pharmaceutical sciences and the industry, by the very nature of their operations and procedures, use a combination of most of the more fundamental disciplines in the development and production of medicinals and medicinal products. Since radioisotopes are so versatile and since they have found extensive use in the fundamental disciplines, they are particularly applicable to pharmaceutical operations and hold particular promise for future applications. The way is clear and the pattern has been set for the future use of radioactivity as a tracer tool in research; however, the use of radioisotopes in industrial processes is still to be exploited and herein lies the next great area of benefit to be gained by evaluation of potentialities. Space does not permit a detailed and lengthy discussion of the potential in this and other areas; however, reference is again made to a detailed study of future potentialities with special emphasis on applied industrial applications which has just been published in the form of an Atomic Energy Commission document (11). Examples of the scope of uses to be developed and exploited may be broadly categorized as follows: (*a*) nondestructive testing (production and quality control); (*b*) thickness gauging and control; (*c*) medicament and package content monitoring; (*d*) level measurements, including control of container fill with liquids and solids; (*e*) moisture measurements and control; (*f*) density measurements and control; (*g*) industrial tracers (studies of fundamental manufacturing processes); (*h*) product contamination, monitoring, and analysis; (*i*) identification analysis; (*j*) automation applications; (*k*) interface detection; (*l*) container-flaw inspection; (*m*) automatic analyses; (*n*) elimination of static charges; (*o*) sterilization, preservation, and pasteurization; (*p*) acceleration of product aging; (*q*) inactivation of enzymes, viruses, etc.; (*r*) plant, viral, fungal, and bacterial mutations; (*s*) irradiation alteration of physical properties; and (*t*) induction of chemical, biological, and enzymatic reactions.

Taking one of these for the purpose of an illustrative example, the area of induction of chemical and biological reactions is intriguing. Ionizing radiation can cause the formation of excited states in matter and thus give rise to

reactive chemical species. The primary species which may be generated are ions, free radicals, and other excited forms which, under certain conditions, will give rise to a variety of reactions, many of which should be of value to the pharmaceutical industry. Another effect, which is called cross-linking, can be induced in some plastic and plastic-like materials. The molecules which are usually in the form of long chains, become attached to each other where they cross and thus the properties of the material are changed, bringing about different physical properties. In this manner new and useful materials for product formulation and other applications may be produced. Such treatment of natural oils, gums, proteinaceous materials, and certain synthetic substances may result in an upgrading of properties to the point of producing new uses for such substances. Studies have been carried out which show that the production of ergosterol can be increased four to five times by radiation treatment of selected yeasts. Yields of the biological synthesis of citric acid can apparently be increased by this procedure as well. These applications point up possible applications to fermentation procedures used in the industry, such as the biological synthesis of antibiotics, steroids, the production of dextrins from starch, and the enzymatic hydrolysis of proteins. These are but a few ideas of the potentialities of radioisotopes for the induction of chemical and biological reactions.

From the standpoint of new research applications, we are seeing many new approaches and concepts of development, resulting from new discoveries and new instrumentation. Large volume, whole body, liquid scintillation detectors are capable of detecting in a short time (two minutes or less) natural levels of radioactivity in man, large animals, and inanimate objects up to 500 lb. in weight. These devices have certain advantages which open the way to new research opportunities not thought possible before. Some of these advantages may be listed: (*a*) natural levels can be detected, (*b*) short counting time, (*c*) small tracer doses required, (*d*) nondestructive analysis, and (*e*) adaptable to continuous process systems. From the pharmaceutical sciences viewpoint there are a number of applications of this technique which are apparent. The effect of environment, nutrition, drugs, and other substances on human and animal body composition can be observed by making possible the nondestructive analysis of the lean-fat ratio, water content, muscle mass, and other bodily components. With minute quantities of tracer dose it will be possible to determine the biological half time of drugs and other materials in large animals and man and to make determinations of absorption, retention, and excretion a relatively simple matter.

The techniques involved will undoubtedly find extensive application to clinical medicine with diagnostic procedures being of major importance. It has been demonstrated that natural levels of potassium[40] are related to the age of individuals, opening up possibilities of studying a whole host of factors on the aging process including the effect of medicament agents used for therapy of the aged.

These are but a few thoughts on the potential uses of radioisotopes in industry and research but they do emphasize the fact that radioisotopes will find increasing new uses in pharmaceutical research and industry as long as there is anything which is not understood in the development, processing, and use of pharmaceutical materials.

SUMMARY

The foregoing discussion of the applications of radioactive isotopes to the determination of medicament release, absorption, permeability, and penetration; to drug metabolism studies; to plant biogenesis and biochemistry; to synthesis; to pharmaceutical analysis; to applied industrial uses; and to the potential applications for the future have emphasized the uniqueness and versatility of this relatively new tool. The advancements that have been made in the past twelve years using the described techniques have been phenominal, yet the surface of application and use with substantial advantage has just been scratched.

As with any new technique there are difficulties encountered in acceptance and use, and radioisotopes have been no exception in this regard. A number of factors have retarded the extended application of radioisotopes. The lack of trained personnel, the erroneous concept of the exceptional health hazards involved, waste disposal problems, the mistaken idea that ionizing radiation induces radioactivity into materials exposed to it, a concern about government regulations, uncertainty about the position that the Food and Drug Administration might take on approval of products exposed to ionizing radiation, and an assumption of high cost of utilization have all been factors. These seeming drawbacks and difficulties to the use of radioactive isotopes and radiation in the pharmaceutical industry are more

apparent than real and we can expect more complete acceptance of isotope applications and uses as soon as the industry as a whole is made fully aware of the potentialities.

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Bioflavonoids in Experimental Ulceration

By EUGENE E. VOGIN and G. VICTOR ROSSI

The bioflavonoid compounds—hesperidin methyl chalcone, orange bioflavonoid complex (OBC) D-2742, OBC 291-R-1, and OBC 291-Y-1, administered alone or in combination with ascorbic acid, were evaluated for antiulcer activity according to five different procedures for producing experimental ulcers: (I) depot histamine-induced gastric ulcers in the guinea pig, (II) reserpine-induced gastric ulcers in the seven and one-half hour pylorus ligated rat, (III) thermally induced serosal burn lesions in the rat stomach, (IV) ulcers induced by fifteen-hour pylorus ligation in the rat, and (V) gastric ulceration in the rat induced by repeated injections of reserpine. The combination of OBC 291-R-1 and ascorbic acid exhibited a greater trend than either agent alone in reducing the incidence of ulceration in procedure I. OBC 291-Y-1, ascorbic acid, and combinations of these two factors reduced the incidence of ulceration in procedure II. Neither the bioflavonoid compounds nor ascorbic acid alone or in combination were effective in reducing the degree of ulceration in the other three experimental procedures.

SUBSEQUENT to the announcement by Szent-Gyorgyi and his co-workers (1–3) of the extraction of an active flavonoid principle from citrus fruit, there have been many contradictory reports relevant to the ability of bioflavonoid substances to modify capillary fragility and permeability. Although several studies have provided data which indicate that bioflavonoids are of value in maintaining capillary integrity, definitive evidence of such activity remains to be established. In 1937, Bentsath and Szent-Gyorgyi (4) reported that traces of ascorbic acid were required for citrin activity. Since that time, a considerable amount of evidence has been adduced to support the concept of a relationship between the flavonoids and ascorbic acid (5). For example, it has been shown that flavonoids protected ascorbic acid against oxidation both *in vitro* (6, 7) and *in vivo* (8, 9), and that ascorbic acid exercised a similar protective effect on flavonoids (10).

Capillary integrity being vital to the normal function of mucous membranes, experimental and clinical lesions of the gastrointestinal mucosa have been employed by several investigators in evaluating the biological activity of flavonoids. LaBarre and Desmarez (11) reported that khellin protected pylorus-ligated rats, rabbits, and dogs from ulceration. These investigators were also able to reduce the incidence of gastric hemorrhage and erosion in rats subjected to daily intramuscular injection of phenylbutazone by concomitant administration of khellin. The data of Rossi and his associates (12) indicated the possible existence of a synergism between ascorbic acid and hesperidin in the prevention of histamine-induced gastric mucosal damage in guinea pigs. DiMaggio (13) similarly demonstrated in guinea pigs that quercetin reduced the severity of gastric ulcers evoked by histamine. Weiss, *et al.* (14, 15), reported that a water-soluble flavonoid complex in combination with ascorbic acid[1] was of value in the treatment of hemorrhage associated with gastric and duodenal ulcers in humans.

Based largely on the hypothesis that vascular disturbances in the mucosa of the stomach constitute a major factor in the pathomechanism of gastric ulceration, it was the purpose of this investigation to evaluate several flavonoid substances for their ability to modify the incidence or degree of ulceration induced by various experimental methods. It was further intended that this study would provide additional data on the synergism between ascorbic acid and bioflavonoids which has been demonstrated in other biological systems.

EXPERIMENTAL

I. **Depot Histamine-Induced Gastric Ulcers in the Guinea Pig.**—English, short hair, male guinea pigs of 250 to 350 Gm. body weight were employed

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[1] Trade-named C. V. P.

throughout this phase of the study. These animals were maintained in individual cages in constant temperature quarters. Gastric ulcers were produced according to the Harrisson, *et al.* (16), modification of the Hay, *et al.* (17), technique. All animals received a daily intramuscular injection (into the back musculature) of 1.0 ml./Kg. of "depot histamine" fifteen minutes after subcutaneous injection of 2 mg./Kg. of diphenhydramine hydrochloride. "Depot histamine" was prepared by suspending 25 mg. of histamine diphosphate and 5 mg. of diphenhydramine hydrochloride in 1.0 ml. of a mixture containing beeswax and mineral oil in a ratio of 1:3.4. This procedure was continued for ten successive days after which all surviving animals were sacrificed; their stomachs were removed and examined. The degree of gastric ulceration was graded according to a modification of the characterization suggested by Keyrilainen and Passonen (18), which takes into consideration both the number and size of the ulcers (Table I). Animals which died during the experimental period were autopsied as soon after death as possible.

TABLE I.—ULCER SCORING SYSTEM

Ulcer Score[a]	Index of Mucosal Damage
0	0
<4	2
4–9	4
10–19	6
20–39	8
>39 (or perforated)	10

[a] Ulcer score = total number of ulcers (gastric and esophageal) + total length of all ulcers in millimeters (greatest dimension of each ulcer).

On the basis of preliminary studies which determined the amount of milled Rockland guinea pig diet consumed by each animal per day, ascorbic acid and/or orange bioflavonoid complex (OBC) 291-R-1[1] were incorporated in the diet in concentrations calculated to provide the average daily intakes indicated in Table II. Each group of animals was permitted to consume its respective prepared diet *ad libitum* for fourteen days prior to and ten days during the course of depot histamine injections. The control group received only milled diet.

Although the differences in the mean index of mucosal damage among the treated and control groups were insufficient to establish a statistically significant degree of protection against histamine-induced ulcers, comparison of ridit values (19) indicated that the combination of OBC 291-R-1 (20 mg./day) and ascorbic acid (100 mg./day) exhibited a greater trend than either agent alone in reducing the severity of ulceration. It may also be noted that the protection provided by this combination appeared to be somewhat greater than that offered by a much larger dose (50 mg./day) of the bioflavonoid alone.

Dale and Laidlaw (20) and, more recently, Kowalewski (21) have advanced the vascular reaction to histamine as a primary factor in the pathogenesis of gastric ulcers. Although the mode of action of the bioflavonoids and ascorbic acid in reducing gastric mucosal damage remains largely obscure, experimental evidence suggests that they function to maintain capillary integrity in the presence of histamine insult.

[1] The bioflavonoid materials used in these studies were generously supplied by Dr. Glenn H. Joseph, Research Department, Sunkist Growers, Corona, Calif.

TABLE II.—ORANGE BIOFLAVONOID COMPLEX 291-R-1 AND ASCORBIC ACID IN THE DEPOT HISTAMINE-TREATED GUINEA PIG[a]

Treatment	Average Daily Intake, mg.	N[b]	Mean Index of Mucosal Damage	Ridit
Control	..	18	7.5	0.50
OBC 291-R-1	20	19	8.4	0.55
OBC 291-R-1	50	16	5.2	0.38
Ascorbic acid	100	11	8.2	0.54
OBC 291-R-1 and ascorbic acid	20 100	12	4.0	0.30

[a] All animals received daily i. m. injections of "depot-histamine" for a period of ten days.
[b] *N* = Number of animals surviving ten-day experimental period. Each group consisted initially of 20 animals.

II. **Reserpine-Induced Gastric Ulcers in the Seven and One-Half Hour Pylorus-Ligated Rat.**—Male albino Sherman rats (Scott Farms), weighing from 150 to 225 Gm., and housed in individual cages in constant temperature quarters were used throughout these studies. The ulcerogenic procedure was basically that described by Leusen and Lacroix (22), in which reserpine (5 mg./Kg., i. p.) was administered to ether-anesthetized rats immediately following ligation of the pylorus according to the technique of Shay (23). Seven and one-half hours after the operative procedure, all surviving animals were sacrificed; their stomachs were removed, graded for ulceration according to the system previously outlined (Table I), and the volume, pH, free and titratable acidity of the gastric contents were determined.

Ascorbic acid and OBC 291-Y-1 were incorporated in milled Rockland rat diet in amounts calculated to provide the approximate daily intakes listed in Table III. The rats were permitted to consume their respective diets *ad libitum* for fourteen days prior to the surgical procedure. Control animals were fed only milled Rockland rat diet.

Orange bioflavonoid complex 291-Y-1 alone, ascorbic acid alone, and combinations of these two factors reduced the mean degree of gastric ulceration in reserpinized seven and one-half hour pylorus-ligated rats (Table III). Inspection of ridit values indicated a trend in the direction of protective activity for these substances alone and in combination, however statistical analysis of the results revealed probability values exceeding the 5% level in each group. Analysis of gastric contents for volume, pH, free and total titratable acidity did not indicate any significant differences among treated and control animals.

Considerable attention has been devoted in recent years to the correlation of the pharmacologic activity of reserpine and its ability to alter tissue levels of 5-hydroxytryptamine (5-HT). Blair, *et al.* (24), have demonstrated that iontophoretic administration of lysergic acid diethylamide, a potent inhibitor

TABLE III.—ORANGE BIOFLAVONOID COMPLEX 291-Y-1 AND ASCORBIC ACID IN THE RESERPINIZED[a] SEVEN AND ONE-HALF HOUR PYLORUS-LIGATED RAT

Treatment	Average Daily Intake, mg.	N[b]	Mean Index of Mucosal Damage	Ridit	Gastric Volume, ml.	pH	Free[c] Acid	Total[c] Acid
Control	...	10	6.7	0.50	7.7	1.5	7.2	10.8
OBC 291-Y-1	20	10	6.4	0.44	7.0	1.5	6.3	9.3
OBC 291-Y-1	50	9	4.1	0.29	7.4	1.7	7.2	11.0
Ascorbic acid	100	9	4.3	0.34	7.3	1.6	6.3	9.1
OBC 291-Y-1 and ascorbic acid	20 100	10	4.6	0.32	7.1	1.6	5.9	9.1
OBC 291-Y-1 and ascorbic acid	50 100	10	5.1	0.29	7.1	1.5	7.6	10.4

[a] All animals received 5 mg./Kg. reserpine i. p. immediately after pylorus ligation.
[b] *N* = Number of animals surviving the seven and one-half hour experimental period. Each group consisted initially of 10 animals.
[c] Ml. 0.1 *N* hydrochloric acid.

of 5-HT, increased the number of petechiae resulting from the application of negative pressure to the skin of humans. Bracco, *et al.* (25), reported an increase in capillary resistance in rats, guinea pigs, and humans following the injection of 5-HT. These studies provide data which suggest that 5-HT may play a role in the maintenance of capillary integrity. Although this concept remains to be definitively established, it is nevertheless interesting to speculate that the bioflavonoids and ascorbic acid inhibit the vascular reactions resulting from reserpine-induced depletion of 5-HT, or that they may function to maintain capillary resistance despite loss of 5-HT.

III. **Thermally-Induced Serosal Burn Lesion in the Rat Stomach.**—Rats were anesthetized with hexobarbital sodium (100 mg./Kg., i. p.) and their stomachs exposed by a small mid-line abdominal incision. A burn lesion was produced by thermocautery[3] about 4 mm. below the transverse ridge of the posterior wall of the glandular portion of stomach, according to the procedure outlined by Skoryna and his associates (26). These investigators contend that the advantages of thermal injury reside in production of a lesion involving the entire thickness of the gastric wall, in the relatively long persistence of the injury, and in the histological resemblance during the healing phase to that of healing in a human penetrating ulcer.

For seven days preceding and eight days after induction of the gastric lesion, the animals were permitted free access to milled Rockland rat diet in which was incorporated: group 1, hesperidin methyl chalcone, 100 mg./day; group 2, ascorbic acid, 100 mg./day; group 3, hesperidin methyl chalcone, 100 mg./day, and ascorbic acid, 100 mg./day; group 4, control, no drug. Each group consisted of 20 animals. At the termination of the fifteen-day experimental period, all surviving animals were sacrificed, their stomachs removed, and the lesions graded. Gross examination of the lesions sustained the findings of Skoryna, *et al.* (26), as to the characteristics of the serosal burn. In each case there was a definite deep ulceration, the base of which was the serosa, which exhibited areas of necrosis, a layer of granulation tissue with collagen fibers, and beginning orientation of the vascular network.

[3] Wappler Thermocautery, American Cystoscope Makers, Inc., Pelham Manor, N. Y.

Analysis of the results of this investigation showed that, in regard to severity of ulceration, there was no significant difference between treated and control animals, or between the individual agents, in the dosage ranges employed. The failure of hesperidin methyl chalcone, ascorbic acid, and combinations of these agents to favorably influence the healing rate of the thermally-induced serosal lesion in the rat stomach may be at least partly attributed to the rapid rate of spontaneous healing evident in "normal" animals. Although the possibility was not investigated, a species such as the guinea pig maintained on an ascorbic acid- and flavonoid-free diet might constitute a more sensitive substrate in this assay.

IV. **Ulcers Induced by Fifteen-Hour Pylorus Ligation in the Rat.**—Employing the technique of Shay (23), pylorus ligation was performed in several groups of ether-anesthetized rats. Fifteen hours after the operative procedure, all animals were sacrificed, their stomachs removed, examined for ulceration according to the criteria previously described (Table I), and the volume, pH, free and total titratable acidity of the gastric contents were determined. This experimental procedure followed a fourteen-day period during which the following compounds were administered *per os*, twice daily: group 1, OBC D-2742, 20 mg./Kg.; group 2, OBC D-2742, 50 mg./Kg.; group 3, OBC 291-R-1, 20 mg./Kg.; group 4, OBC 291-R-1, 50 mg./Kg.; group 5, ascorbic acid, 100 mg./Kg.; group 6, OBC D-2742, 20 mg./Kg., and ascorbic acid, 100 mg./Kg.; group 7, OBC 291-R-1, 20 mg./Kg., and ascorbic acid, 100 mg./Kg.; group 8 control, 2 ml. distilled water *per os*, twice daily. Each group consisted of 20 animals with the exception of the control group which contained 35 rats.

Comparison of the degree of ulceration revealed no significant differences among those animals which received ascorbic acid, bioflavonoids OBC D-2742 and OBC 291-R-1, combinations of ascorbic acid and bioflavonoids, and those animals which served as controls. Furthermore, analysis of the gastric contents for volume, pH, and acidity did not indicate any significant differences among these factors in the various groups of treated and control animals.

Goldberg (27) has summarized the various factors which contribute to the development of gastric

ulcers following ligation of the pylorus. Distention of the stomach by accumulated gastric secretions was considered to be an important contributing factor. This is consistent with the observations of Shay, *et al.* (23), who reported that the appearance of ulcerative lesions after pylorus ligation depended on the presence within the stomach of a sufficient volume of gastric fluid for a sufficient length of time. It may be that a diminution of capillary integrity in the gastric mucosa is not a dominant factor in the genesis of ulcerative lesions subsequent to ligation of the pylorus. On this basis, the pylorus-ligated animal would not appear to be a desirable preparation for the evaluation of the antiulcer activity of substances which act primarily by increasing tissue resistance rather than by inhibiting gastric secretion.

V. **Gastric Ulceration in the Rat Induced by Repeated Injections of Reserpine.**—Utilizing a modification of the method of LaBarre (28), gastric lesions were produced in rats by daily intramuscular injections of reserpine (1 mg./Kg.) for ten successive days. For fourteen days prior to the onset of reserpine administration and during the course of alkaloid injections the animals in this study were given free access to milled Rockland rat diet in which was incorporated: group 1 OBC 291-Y-1, 50 mg./day; group 2, ascorbic acid, 100 mg./day; group 3, OBC 291-Y-1, 50 mg./day, and ascorbic acid, 100 mg./day; group 4, control, no drug. Each group consisted of 20 animals. At the conclusion of the experimental period all surviving animals were sacrificed, their stomachs excised, and the extent of ulceration graded according to the scheme outlined in Table I.

Neither OBC 291-Y-1, ascorbic acid, or the combination of these agents in the concentrations employed significantly reduced the incidence or severity of gastric mucosal damage produced by repeated injections of reserpine. Reserpine has been shown to increase the volume and free acidity of gastric secretion in man and in animals (22). This secretory activity is evidently a primary factor in the development of hemorrhagic erosions in the gastric mucosa of the rat following repeated administration of reserpine, and for the absence of significant protection in the case of ascorbic acid-bioflavonoid treatment.

SUMMARY

The ability of certain bioflavonoid compounds to modify lesions of the gastric mucosa produced by various experimental methods was investigated.

No acceleration of the spontaneous healing of thermally induced lesions of the stomach wall in rats was obtained by treatment with the bioflavonoids.

On the basis of the parameters employed in this study, the bioflavonoid compounds exhibited no activity in those procedures in which gastric acid hypersecretion or distention of the stomach constitute primary ulcerogenic factors.

Administration of bioflavonoid alone, ascorbic acid alone, and combinations of these agents provided indicative, but not statistically significant, protection against reserpine-induced gastric ulcers in the seven and one-half hour pylorus-ligated rat.

Although histamine is a potent secretory stimulant, the effect of this amine on capillary integrity has been cited as a major factor in its ulcerogenic action. In this study, a greater degree of protection against depot histamine-induced gastric mucosal damage in the guinea pig was obtained with a combination of bioflavonoid and ascorbic acid than with either agent alone.

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Essential Oil Synthesis in Mint

By R. H. REITSEMA†‡, F. J. CRAMER†, N. J. SCULLY§, and W. CHORNEY§

Labeling peppermint oil by growth of the plants under an atmosphere containing $C^{14}O_2$ has permitted study of some phases of the biochemistry of essential oils. It has been found that oil is synthesized from carbon dioxide in detectable amounts in less than fifteen minutes. Production of peppermint oil continues for several hours in the dark and is not stopped by cessation of photosynthesis. The loss of oil from the plant to the air is very large. Infiltration experiments were investigated as an alternate way to introduce radioactivity. Menthol was obtained from menthone. Several compounds, including oxygenated terpenes, were produced from terpene hydrocarbons.

RADIOCARBON tracer techniques provide a method for solving many problems of interest in terpene chemistry. Such problems include the time required for synthesis of essential oils, the order of formation of various constituent compounds, and the rate of loss of oil from growing plants to the atmosphere. The present study describes the development of some methods of radiochemical study of essential oils and some information obtained in preliminary work which is correlated with previously obtained information.

EXPERIMENTAL PROCEDURES

$C^{14}O_2$ Fixation by Plants.—Initial experiments were conducted to determine the best methods of handling the plant material, the optimal specific activity of atmospheric carbon for production of useful levels of radioactivity in biosynthesized oil, the required length of photosynthesis or exposure of the plant to $C^{14}O_2$, and the length of time required to obtain a useful autoradiograph of the oil constituents on a chromatoplate. In these studies it was determined that an actively growing leafy shoot of as low as 1.5 Gm. fresh weight was adequate experimental material. With a three-hour light-exposure period, a radiocarbon activity level of 10–50 μc. per mg. of atmospheric carbon was adequate, but a three-minute photosynthesis period required as much as 200 μc. per mg. atmospheric carbon. A summary of the experiments is given in Table I.

Actively growing, leafy stem cuttings of greenhouse grown peppermint (*Mentha piperita*) plants were used in all experiments. The cuttings consisted of the terminal portions of either main or lateral stems bearing three to five pairs of opposite leaves. They were recut under water to avoid wilting, and during the experimental period the bases of the stems were kept submerged in water. For exposures of a week's duration a $^1/_4$-strength complete nutrient solution was used.

Most of the experiments were conducted in a controlled-environment room maintained at 75 ± 1° F. with 1,200 f.-c. of light supplied by fluorescent tubes and incandescent lamps. The short term experiments, three to fifteen minutes in duration, were conducted under greenhouse conditions with full sunlight intensity of 7,000 f.-c.

The mint cuttings were placed in a closed system having a volume of 1–101 L., depending upon the objectives of the experiment and its duration. The 1-L. system consisted of a single flask and was used for very short-term $C^{14}O_2$ fixation studies. The larger system, used for the longer-term fixation studies, consisted of a flask to contain the plant material, flasks for generating the carbon dioxide, a series of air reservoir flasks, an air circulating pump, and an ionization chamber for continuous measurement of the $C^{14}O_2$ content of the system. Known amounts of $C^{14}O_2$ were released into the system by treating $BaC^{14}O_3$ with dilute lactic acid. The carbon dioxide concentration varied in these fixation experiments from 0.03 to 0.12%.

To prepare randomly labeled oil constituents (Experiment 4), rooted cuttings were grown in the 101-L. system for seven days (sixteen hours light and eight hours dark) at an average specific activity of 12 μc. per mg. of atmospheric carbon.

Isolation of Essential Oil.—At harvest, the cuttings were fractionated into leaves and stems, and, since the greater part of the oil is found in leaves, they alone were subjected to distillation. The fresh leaves were ground in a mortar with a few milliliters of water and hexane, transferred to a distilling flask, and distilled slowly. The distillate containing the essential oil was collected in a small volume of toluene.

All assays for C^{14} activity in oil and its constituents were performed in a liquid scintillation counter.[1] The oil constituents were separated by chromatography using the chromatoplate technique of silicic acid-coated glass plates and developing with 10% ethyl acetate in hexane (1). Autoradiographs of the chromatograms were obtained by exposing them to Kodak No-Screen X-ray film for varying periods of time. Although a useful autoradiograph of a chromatographed oil could be obtained in one hour with high activity levels in the oil, as long as eighty-nine days was required with the oils of low activity that resulted from photosynthetic periods of a few minutes duration even with exposure to the highest used specific activity of atmospheric carbon dioxide.

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[1] Tri-Carb Counter, Packard Instrument Co., Lyons, Ill.

TABLE I.—SUMMARY OF EXPERIMENTS

Experiment No.	Weight of Leaves, Gm.	Volume of System, L.	$BaC^{14}O_3$, mg.	Radioactivity of Atmosphere, μc./mg. C	Exposure Time	Radioactivity of Oil, Disintegrations/min.
1	1.72	22.2	0.8[a]	2.2	3 hr.	20,000
2	1.59	22.2	1.6[b]	4.9	3 hr.	2,615
	1.92				3 hr.[f]	9,180
	1.92				3 hr.[g]	3,520
3	1.50	22.2	2.3	10	16 hr.	
4	7.9	101	101.7	12	7 days	124×10^6
5	1.6	9.75	1.5[b]	10	20 min.	20,000
6	1.75	9.75	1.7[b]	10	1 hr.	37,835
7	1.6	22.2	1.74[c]	10^d	3 hr.	119,000
	1.55				3 hr.[h]	146,000
	1.6				3 hr.[i]	146,000
8	1.6	22.2	17.3[c]	50[e]	3.5 hr.	1.3×10^6
	4.05				3.5 hr.[j]	6.6×10^6
9	2.15[a]	1	1.1[c]	200	3 min.	1,618
10	1.9[a]	1	1.0[c]	196	7 min.	2,197
11	2.05[a]	2.3	1.9[c]	207	15 min.	39,908

[a] Includes weight of stems. [b] 10.65 μc./mg. $BaCO_3$. [c] 40.8 μc./mg. $BaCO_3$. [d] At 0.06% carbon dioxide initially. [e] At 12% carbon dioxide initially. [f] Followed by three hours in the dark in normal air. [g] Followed by five hours in the light in normal air. [h] Followed by three hours in the light in normal air. [i] Followed by three hours in the dark in normal air. [j] Followed by forty-eight hours in normal air.

Infiltration Experiments.—These experiments were conducted to trace the interconversions that took place when labeled oil constituents were applied to mint leaves. After separation by chromatography, the desired C^{14}-labeled compound was cut from the chromatoplate and eluted from the adsorbent material with toluene. The toluene was evaporated and the radioactive compound was redissolved in hexane and applied as a thin film to the upper surface of a mint leaf on an intact plant. After three hours in light under greenhouse conditions, the leaf was harvested and assayed as previously described.

Oil Loss by Mint Plants.—A group of cuttings were allowed to fix $C^{14}O_2$ for three hours. At the end of this period, one-third of the cuttings were harvested and the radioactivity of the oil fraction was determined. The other two-thirds were allowed to grow in a normal atmosphere for twenty-three hours. During this time period, air was circulated through the system. Included in the system was a cold trap containing toluene to trap the volatile oil lost by the plants to the surrounding atmosphere. The quantities of oil were estimated from determinations of the radioactivity of the toluene solution.

RESULTS AND DISCUSSION

Time for Oil Synthesis.—Essential oils have been commonly thought to be end products of a long series of reactions. They would, therefore, be produced more slowly than other photosynthetic products. The formation also would be expected to be slow if the oils were simply by-products of normal plant photosynthesis. Comparative analyses of oils harvested at the start and the end of the growing season regularly show large differences between oils from young, actively growing plants and from mature plants. This difference also has been observed between young and mature leaves on the same plant (2). This might suggest a slow conversion of constituents throughout the growing season. Laboratory results using labeling techniques lead to completely different conclusions. Initial exposures of three hours proved to be very long for oil synthesis (Experiments 1, 2). These oils from three-hour labeling experiments appeared to be uniformly labeled. They were not significantly different from oil labeled for seven days (Experiment 4). A sixteen-hour exposure to low activity gave an autoradiograph in five days which showed the presence of radioactive peppermint constituents, i. e., menthol, pulegone, menthone, and hydrocarbons (Experiment 5).

The rate of oil formation was determined by reducing the length of exposure of the plant to the $C^{14}O_2$ atmosphere. Photosynthetic periods of three, seven, and fifteen minutes were used in one series of experiments (Experiments 9, 10, and 11). Only a small portion of the radioactivity is found in the oil, particularly in these short-term experiments, and it was necessary to run a greater concentration of radiocarbon and to run autoradiographs for several weeks. The results of the autoradiograph are shown in Fig. 1. Only one spot was visible in oil from the three-minute exposure. It appeared on the chromatoplate at R_f 0.77. Oil from seven-minute labeling gave radioactive spots at R_f 0.77 again, and also at 0.23 and 0.35. The fifteen-minute exposure produced oil which after chromatography showed radioactive spots at the same 0.77, 0.23, and 0.35 areas, but also at areas corresponding to major peppermint constituents, such as R_f 0.30 for menthol, 0.42 for pulegone, 0.49 for menthone, and 0.90 for unresolved hydrocarbons. Thus a sequence of formation of components of the oil is evident. The labeling and identification techniques used are adequate to show the formation of peppermint oil within fifteen minutes. This is a maximum amount of time for the formation of peppermint constituents from carbon dioxide.

The experiments were not sufficiently definitive to clarify the sequence of formation of peppermint oil constituents. The compounds located on the chromatoplate at R_f 0.23 and 0.77 are of special interest, particularly the latter. Piperitone is present in the seven-minute oil and is dominant in the fifteen-minute oil. Its existence as an early constituent of peppermint has been anticipated by other work (3). Judged by the relative intensity of

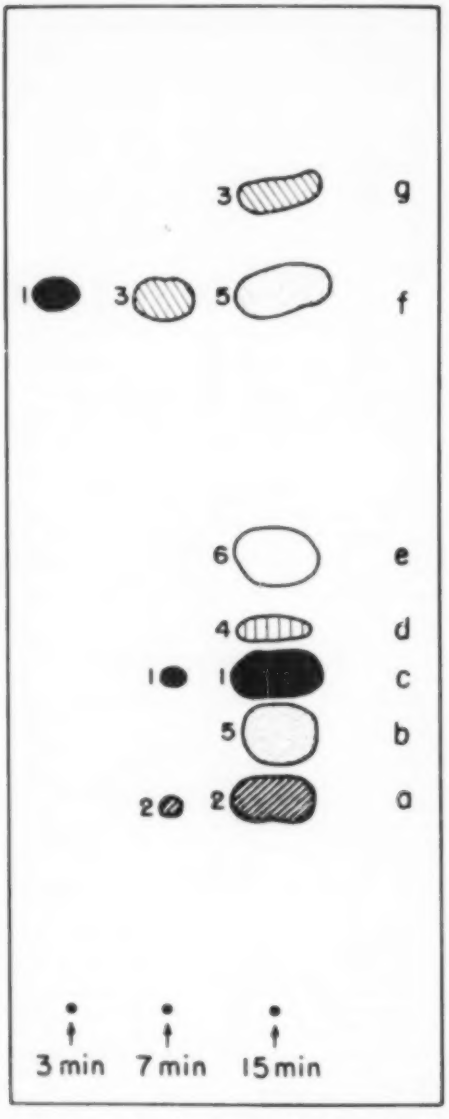

Fig. 1.—A drawing representing an autoradiograph of chromatogram of oils produced by mint plants exposed to $C^{14}O_2$ atmosphere for three, seven, and fifteen minutes. Although the spots were quite distinct, the contrast in the spots was inadequate for reproduction. *a*, Unidentified spot at R_f 0.23; *b*, menthol; *c*, piperitone; *d*, pulegone; *e*, menthone; *f*, unidentified spot at R_f 0.77; *g*, hydrocarbons. The numbers 1 to 6 indicate relative intensity of the spots, which decreases with increasing number.

the radioactivity it can be suggested that menthol follows the appearance of pulegone, menthone, and hydrocarbons. The detection method is not quantitative and the presence of a labeled component may indicate concentration rather than chronology.

Nonphotosynthetic Oil Productions.—A study was made to determine whether mint plants continue to produce oil after cessation of photosynthesis (Experiments 2, 7). Mint cuttings of nearly equal weight and leaf surface were exposed to $C^{14}O_2$ (10 μc. per mg. atmospheric carbon) for three hours of light. At the end of this period, one-third of the cuttings were harvested and the amount of activity in the oil fraction was found to be 119,000 c. p. m. Another third was allowed to continue photosynthesizing in a normal carbon dioxide atmosphere for three additional hours, and the other third was kept in dark for three hours in a normal carbon dioxide atmosphere before determining the activity in the oil. In the latter two samples the counts of the oil had increased 22.7%, to 146,000 c. p. m. In another experiment (Experiment 8) the plants were allowed to metabolize for forty-eight hours in a normal atmosphere after an initial exposure to $C^{14}O_2$ for three hours. In three hours 815,000 c. p. m. per Gm. of leaf appeared in the oil fraction; after forty-eight hours this value was 1,630,000. These results indicate that oil formation can continue without photosynthesis, provided that the necessary precursors are present, for at least forty hours.

The relative rates of oil formation in the light and dark were not conclusively shown by the experiments. Experiment 2 indicated that the plant put in the dark for three hours after exposure to labeled atmosphere produced an oil with three times as much radioactivity as did the plant put in the light. Experiment 8, however, indicated no real difference in a similar experiment. This again demonstrates the need for replicate determinations in plant studies. Further investigation of this phenomenon is in progress.

Infiltration Experiments.—The labeling of the essential oil by growth of plants in an atmosphere of carbon dioxide was appealing because of its simplicity. One inherent difficulty, however, was that the other plant components would be labeled at the same time as the oil and oil intermediates. Feeding labeled components through the root system or through the leaves were alternate methods, but they could lead to problems in interpretations of results, since many reactions might occur which would not be typical of those taking place within the oil gland. With this difficulty in mind, it was decided to infuse labeled components into the leaves.

Labeled menthol infiltrated into the leaves apparently underwent no conversion. The only radioactive spot found on the chromatogram of the resulting oil was at the menthol area itself. Application of menthone, however, resulted in the appearance of the major portion of the activity in the menthol area, with some still remaining as menthone. Lesser amounts of activity were found at the pulegone area and at the hydrocarbon area. This correlates partially with other observations that menthol is produced from menthone. Limonene and pinene gave oils with five radioactive spots. The formation of these additional materials at the menthol, pulegone, and menthone areas suggests that terpene hydrocarbons are undergoing many interconversions including oxidation. This type of oxidation is observed *in vitro* also. Whether these

are normal paths of oil synthesis or of autoxidation is not determinable from the infiltration technique.

Oil Loss by Mint Plants.—The amount of oil lost from a mint plant has practical significance. The intensity of the odor in the neighborhood of a mint field would suggest that this is an appreciable quantity. Although the simulation of a natural growth situation was not attempted, an idea of the quantity of oil lost was obtained by radiocarbon studies. Mint cuttings were allowed to fix $C^{14}O_2$ (50 μc./mg.) for three hours in the light (Experiment 8). One-third of the plants were harvested and the amount of activity in the oil determined. The other two-thirds were allowed to metabolize in a normal atmosphere and the oil that volatilized was collected in a toluene trap. This oil was found to contain 1% of the activity of the oil originally present in the leaves. Chromatography of this oil showed that it was essentially the same as the oil in the plants. Metabolism of oil during the experiment was not measured and the value obtained simply indicated that a very significant amount of oil was lost continuously by evaporation.

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Substitute for Cocoa Butter as a Suppository Base in Tropical Countries

By J. S. ROBERTSON

Cocoa butter, which is still widely used as a suppository base, has several well-known defects, such as slowness to harden if overheated and the assumption of solid forms with low melting points. In tropical countries, a further disadvantage is the proximity of its melting point to average room temperature. The seed fat from a species of *Shorea*, having a higher melting point, seemed a possible substitute. Its behavior on heating and cooling is described and the results indicate that it has certain advantages over cocoa butter where relatively high room temperature is a problem.

COCOA BUTTER is widely used in tropical countries as a suppository base, but its usefulness is lessened by the fact that its melting point lies close to the average room temperature of 28°, with the result that suppositories made from it melt on handling. Sometimes attempts are made to overcome this defect by adding beeswax, but this procedure has two disadvantages; it entails considerable overheating of the cocoa butter, and the mass on cooling may not be homogeneous. Because of these recognized defects, more commonly, suppositories are made without the addition of beeswax, and a solid product is maintained by cooling in a refrigerator.

It was thought that some readily available natural fat with a melting point just below body temperature and well above ambient temperature, but possessing the otherwise acceptable properties of cocoa butter, would be more satisfactory in giving products that would withstand moderate handling without special precautions.

Of the available materials, the published data (1) indicated that Borneo tallow would appear to meet the essential requirements. Borneo tallow also known in commerce as illipe butter, is the fat obtained from the seeds of certain species of *Shorea*, family *Dipterocarpaceae*. The fats used in this work were obtained from the seeds of *Shorea gysbertsiana* Burck, *Shorea martiniana* Scheff, *Shorea palambanica* Miq. (all from Borneo), and *Shorea sinkawang* (from Malaya). Table I gives a description of these fats.

EXPERIMENTAL

Extraction of the Fats.—The seeds were shelled and the flesh grated by hand. Mechanical disintegration was attempted but resulted in a sticky paste due to the high fat content and the heat generated during the process. The grated material was extracted with iso-hexane (b. p. 56–70°) in a Soxhlet

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TABLE I.—DESCRIPTION OF THE FATS

	Sh. martiniana	*Sh. gysbertsiana*	*Sh. palambanica*	*Sh. sinkawang*	*Cocoa[a] Butter*
Acid value	1.23	41.2	1.36	1.84	4.0
Saponification value[a]	194	198	185	165	188–195
Iodine value (ICl method)[b]	15.9	15.5	16.0	15.7	35–40
Melting point, °C.[b]	36.6	34.4	34.0	34.2	31–34
Refractive index 40°[b]	1.457	1.455	1.457	1.457	1.456–1.458
Appearance	Yellow-green to brownish-green, the color bleaches on storage				
Consistency	Harder than cocoa butter	Similar to cocoa butter			
Odor and taste	Similar to cocoa butter				

[a] British Pharmacopoeia 1958 values (2). [b] British Pharmacopoeia 1958 methods for cocoa butter (2).

apparatus. After removal of the solvent, 0.1% of propyl gallate[1] was added as antioxidant since, in preliminary experiments, it was found that when no antioxidant was added the fat became rancid.

The high acid value of the fat from *Sh. gysbertsiana* was almost certainly due to its being rancid before extraction. Rancidity was detected in the extracted oil by Kerr's modification of the Kries test (3). Apart from this, the acid and iodine values were much lower than those given by Williams (1).

The properties of the fat from *Sh. martiniana* seemed to be most worthy of further investigation.

Determination of Transition Point.—Preliminary cooling curves were constructed. Approximately 0.5–1.0 Gm. of the fat, after being heated to various temperatures, was placed in a glass test tube (2.5 × 1 cm. external diameter) with a thermometer reading to 0.2° inserted. The thermometer was retained in position with rubber tubing. This assembly with or without a jacket was placed in a cooling bath at −20, 0, or 15°. The fall in temperature was noted at ten-second intervals. The curves obtained showed no inflections and did not give the information desired. Therefore, the procedure was modified to that used by Clarkson and Malkin (4) for their thermal investigation of pure triglycerides. The fat was heated in a controlled temperature water bath to various temperatures above its melting point. One milliliter of fluid fat was placed in a thin-walled glass container and a single-junction, glass-sheathed, copper-constantan thermocouple was immersed in the fat to such a depth that it was centered both vertically and horizontally. The stoppered tube was surrounded by a thin-walled aluminum jacket. This complete apparatus was placed to a constant depth in water at the desired temperature in Dewar flasks. The temperature of the fat could be read to 0.05° by means of a mirror galvanometer. Readings were taken at suitable intervals, usually every thirty seconds. The results are shown in Figs. 1 and 2.

Preparation of Suppositories from Borneo Tallow.—The fat was melted, with stirring, at either 36.7 or 40° and poured into 15-grain, unlubricated suppository molds. The fat which was heated to 40° was first cooled in a water bath at 20° until transition began, and then poured. The poured suppositories were allowed to set at either 0, 20, or 28° (room temperature). At various time intervals

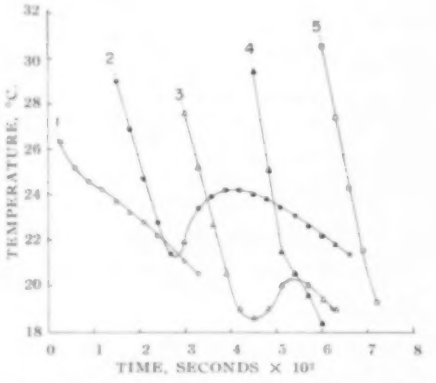

Fig. 1.—Cooling curves of Borneo tallow, cooling bath temperature 0°C. ○, heated to 36.7°; ●, heated to 38°; △, fat heated to 38° and held for one hour; ▲, heated to 40°; □, heated to 42°.

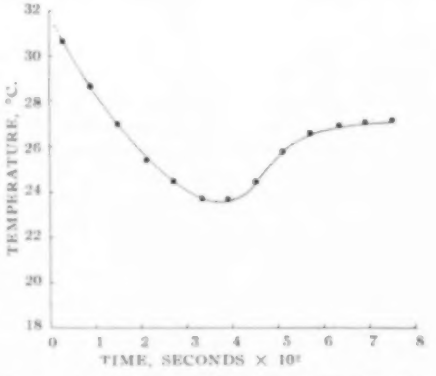

Fig. 2.—Cooling curve of Borneo tallow; heated to 42°, cooling bath temperature 20°.

the suppositories were tested to determine whether the fat had returned to its original melting point. The results shown in Table II were obtained.

Suppositories were also prepared containing 10 and 20% of chloral hydrate. The powdered chloral hydrate (No. 90 sieve) was incorporated into the

[1] Progallin is the trade name of NIPA Laboratories, Ltd., London and Cardiff, for propyl gallate.

TABLE II.—TIME TAKEN FOR FAT TO RETURN TO ORIGINAL MELTING POINT AFTER HEATING AND COOLING

Heated to, °C.	Cooled at, °C.	Returned to Original Melting Point After 1 hr.	3 hr.
36.7	0	No	No
	20	No	Nearly
	28	Yes	Yes
40	0	No	No
	20	No	Nearly
	28	No	Nearly

fluid fat at 36.7–37° and allowed to set at room temperature. After twenty-four hours, the melting point of the suppositories containing 10% w/w chloral hydrate was 34.8°, and they withstood moderate handling. Those containing 20% w/w chloral hydrate melted at 33.1° and were a little softer.

DISCUSSION

From Fig. 1 it can be seen that the shape of the cooling curves depends on the temperature to which the fat was heated, and on the length of time it was held at one temperature. When heated to 38°, the fat on cooling assumed a lower melting point and set at 21.4°, and the heat of transformation was evolved. Since this behavior occurred above 36.7° and below 38°, the transition temperature was taken to be between these two temperatures. When the temperature of the cooling bath was raised to 20° (Fig. 2) the cooling curve of the fat, after being heated to 42°, showed that in this case transformation had taken place, resulting in a form with a melting point of 23.5°, indicating that the shape of the cooling curve was also affected by the rate of cooling.

A consideration of the composition of Borneo tallow and the observed behavior of pure triglycerides affords an explanation of this behavior. According to Hilditch (5), Borneo tallow contains: palmito-stearin 5%, oleodipalmitin 8%, oleopalmitin-stearin 31%, oleodistearin 40%, palmitodiolein 3%, and stearodiolein 13%.

The work of Clarkson and Malkin (4, 6) has shown triglycerides to exhibit polymorphism and to exist in four forms, *viz:* vitreous, α, β' (crystalline, monotropic), and β (crystalline, stable), in order of ascending melting point. Further, oleodipalmitin and oleodistearin have been shown to exist in five solid modifications (7), with the melting points given in Table III.

The vitreous form is obtained by first completely melting the solids, then cooling rapidly. The lower melting point forms are obtained by carefully warming the vitreous form. All the lower melting point forms are metastable, and transition into higher melting point forms can take place in the solid state. The transition has been shown to be slow with unsymmetrical mixed triglycerides, e. g., oleopalmitostearin. Clarkson and Malkin (4) stated that the formation of the higher melting point forms from the molten triglyceride is hastened by the presence of seed crystals of the forms and that the β-form separation occurs with slow cooling from just above the melting point.

TABLE III.—MELTING POINTS OF THE KNOWN POLYMORPHS OF TWO TRIGLYCERIDES[a]

	Vitreous	α	β''	β'	β
Oleodipalmitin	12	21.5	29.0	35.0	37.5
Oleodistearin	23	29.5	37.0	41.5	43.5

[a] Melting points are given in °C.

Applying these findings to the present investigation, it is apparent that the 36.7° curve (Fig. 1) represents the cooling curve of the higher melting point forms of the mixed triglycerides where, due to the incomplete melting, seeds of these forms are already present (the fat is opaque and fluid). The smooth form of the curve is not unexpected, since each form can be considered an "impurity," so that no one member can express individuality in the form of an arrest on the curve. The 38° curve (Fig. 1) shows the effect of completely melting the fat so that no seed crystals are present. Transition occurs if conditions allow crystallization to take place before the vitreous form is obtained. When the fat is held at 38° for one hour (Fig. 1) the extended thermal agitation of the molecules ensures more complete randomization. Upon cooling this melt even a lower melting form separates. By heating to much above the melting point and cooling rapidly, noncrystalline forms are obtained, and no heat of transition is evolved. When the fat is heated to 42° but cooled slowly, formation of the higher melting crystalline form is favored and heat of transition is evolved (Fig. 2).

This interpretation leads to the conclusion that the fat should not be completely melted; should be held at the melting temperature for as short a time as possible; and that whether completely melted or not, it should be allowed to cool slowly.

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Hemolysis of Erythrocytes in Various Iso-osmotic Solutions

By E. R. HAMMARLUND† and KAJ PEDERSEN-BJERGAARD‡

A study has been made of the degree of hemolysis of human erythrocytes that occurs when the red blood cells are incubated in many different pharmaceutically employed iso-osmotic solutions. Many distinct differences were noted which further demonstrate the frequent dissimilarities between iso-osmotic and isotonic concentrations. In addition to the hemolytic differences, one of the other main factors in the consideration of the use of isotonic solutions *versus* iso-osmotic solutions is clearly that of the quantities and proportions of the substances used and the resulting rapidity and ease of dilution of the medicinal solution by the body fluids.

IN A PREVIOUS study of iso-osmotic and isotonic solutions Hammarlund and Pedersen-Bjergaard (1) determined the sodium chloride equivalents for 332 pharmaceutical compounds. These were calculated from experimentally obtained measurements of the lowering of the vapor pressure and the depression of the freezing point of their aqueous solutions. It is well known that solutions of some medicinally used substances such as ammonium chloride, boric acid, ethanol, glycerin, and urea fail to prevent the hemolysis of red blood cells in iso-osmotic concentrations. For such substances that pass through or alter the erythrocyte membrane, the iso-osmotic concentration differs markedly from the isotonic concentration. Husa and co-workers (2–9) have investigated the red blood cell hemolytic activity of a number of solutions in reference to a determination of their van't Hoff *i* values or "isotonic coefficients." However, since the hemolytic activities of a large number of pharmaceutical solutions have not been compared previously on an equal osmolar basis, except those examined by Husa (2–9) and by Setnikar and Temelcou (10), this study was undertaken. The purpose was to determine the degree of hemolysis which results when defibrinated blood is added to solutions in iso-osmotic concentration for 161 substances from our previous study. The items selected were those which are completely soluble to the extent of their iso-osmotic concentrations.

METHOD

The method used is essentially the same as that employed by Husa and co-workers (2–9) and is a modification of the method by Hunter (11). The principal deviations from Husa's method were that iso-osmotic concentrations were employed and an aqueous saponin purum,[1] 100 mg./L., was used as the 100% hemolyzing solution for the erythrocytes instead of 0.1% sodium carbonate solution. Finholt (12) and others used a saponin solution for a standard since the alkalinity of the carbonate solution produces a darker red hemolyzed solution which results in a higher colorimetric reading than would be obtained at the body pH of approximately 7.4. The color given by the hemolysis was determined as oxyhemoglobin and this was done as rapidly as possible following the incubation of the defibrinated blood in the solutions being tested.

Fresh venous blood from human volunteers was drawn into a flask containing glass beads. It was immediately defibrinated by rotating the flask with the beads until the fibrin separated. The blood was poured into a small flask and aerated by rotating gently for five minutes. The blood was shaken gently by swirling immediately before each 0.1-ml. sample was withdrawn with a volumetric pipet. The blood was preserved under refrigeration and was not used if it had been drawn more than twenty-four hours earlier.

The solutions were freshly prepared with distilled water; all chemicals used in the study were identical in purity to those reported in the previous study (1).

Quantitative Determination of the Per Cent Hemolysis.—Ten milliliters of each solution studied was added to each of three centrifuge tubes. Three tubes containing 10 ml. of 0.9% sodium chloride served as the colorimetric blanks. Three tubes containing 10 ml. of saponin solution served as the reference color for complete hemolysis; the colorimetric readings of which were made at the beginning, in the middle, and at the end of the experimental determinations. Exactly 0.1 ml. of fresh defibrinated and aerated blood was added to all tubes by means of a volumetric pipet. The tubes were stoppered and inverted several times. They were placed in a water bath at 25 ± 1° for forty-five minutes and were immediately centrifuged for five minutes at approximately 2,000 r. p. m. The supernate was decanted and its absorbance was determined in an EEL photoelectric colorimeter with a standard green filter. The per cent of hemolysis was calculated by dividing the absorbance reading for each unknown solution investigated by the reading obtained from the average complete he-

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[1] Merck and Co., Rahway, N. J.

molysis times 100%. The instrument was previously standardized to read zero absorbance for the 0.9% sodium chloride blank solutions. The test was repeated on another day using different blood samples and new solutions for each of those substances which gave hemolysis. These two sets of values were then averaged and are reported in Table I.

The pH of each experimental solution was determined prior to the addition of the blood and this value to the nearest 0.1 is included in the results in Table I. After the 1% blood sample was added, the pH changed slightly in the direction of 7.4. The amount of change usually was small and depended upon the buffer capacity of the solution.

If an experimental solution was colored before the addition of blood, then this same colored solution was employed as the blank. A few solutions were too intensely colored for an accurate colorimetric determination of hemolysis and for these the method was modified as follows: after centrifuging down the erythrocytes and ghosts, the colored supernate was decanted; the remaining erythrocytes were then resuspended in normal saline and centrifuged again. The resulting erythrocyte volume was compared to the volume in the standard 0.9% sodium chloride solution for an approximate percentage of cells hemolyzed. In some instances it is possible that the color of the solution and the color of oxyhemoglobin are not exactly additive. However, only three solutions in the study (cupric sulfate, oxophenarsine hydrochloride, and sodium ascorbate) were colored appreciably, and no tests were made to determine if these colors were additive.

The absorbance readings from some solutions resulted in a greater than 100% hemolysis due to such factors as the darkening of the red color in alkaline solution and oxidation or reduction reactions with the blood. These solutions are reported as 100% if there were no unhemolyzed erythrocytes in the sediment.

After this study was completed Ansel and Husa (13) showed that zinc ions precipitate oxyhemoglobin; therefore, data for the zinc compounds are not included in the study. Furthermore, it is possible that other compounds similarly are incompatible with oxyhemoglobin since the compatibility of the compounds in Table I with oxyhemoglobin was not determined. But, any apparent change in the physical appearance of any of the solutions or of the unhemolyzed erythrocytes following incubation is reported in the footnotes for Table I.

RESULTS AND DISCUSSION

Hemolytic Values.—The percentage of hemolysis found for the 161 compounds studied are listed in Table I including the iso-osmotic concentration (1) used for each and the pH of each iso-osmotic solution.

Solutions of 90 substances prevented hemolysis in iso-osmotic concentration and 71 substances showed varying degrees from slight to complete hemolysis. Solutions of the inorganic salts of moderate pH prevented hemolysis of erythrocytes. The carbohydrates and most alkali salts of organic acids also prevented hemolysis. Most inorganic and organic acids and alkalies with more extreme pH values or with marked oxidation or reduction properties usually penetrated or altered the erythrocyte sufficiently to give some degree of hemolysis.

Many amine salts usually of a monovalent anion failed to prevent hemolysis. This is in agreement with the results of the permeability of erythrocytes to weak electrolytes obtained by Jacobs and Stewart (14), Jacobs, Glassman, and Parpart (15), and Davson (16), which is summarized well by Thomasson and Husa (7) and Marcus and Husa (9).

From an examination of the hemolytic data from the many compounds studied in equal osmolar concentrations, it is evident that there is no distinct pattern indicating the predominance of any single main mechanism of penetration of the erythrocyte by the solute and solvent which results in hemolysis of the erythrocyte. It appears to be a combination of factors such as pH, lipoid solubility, the molecular and ionic size of the particles, and the inhibition of cholinesterase in the cell membrane (9), to name a few. There are also other complex forces which may also play a role because of their possible denaturing actions on the plasma membrane proteins: substances such as alcohols and urea are known to rupture hydrogen bonding; low surface tension forces may denature proteins; and acids, alkalies, and oxidizing agents often change the structure of proteins owing, in part, to their effects on benzene ring structures, such as those present in the amino acids tyrosine and histidine. The picture is still incomplete.

Ammonium Compounds.—It is well known that the ammonium salts are an exception to the generally accepted view that iso-osmotic solutions of inorganic salts of monovalent anions are osmotically indifferent to red blood cells.

The results of the several ammonium compounds in this study are presented in Table II for comparative study. The results of ammonium compounds from Cadwallader and Husa (6) are also included.

The erythrocyte membrane is permeable to the anions in the first column (about 100%) and impermeable to those in the second column (0%). Since ammonium sulfate and phosphate protected erythrocytes from hemolysis, Jacobs and Stewart's (14) statement that ammonium salts of weak acids and strong acids will cause hemolysis should be amended to include only strong acids of monovalent anions. It is only necessary for a membrane to be impermeable to a single species of ion, i. e., anion or cation, for it to be impermeable to the salt of which this ion is a part (16).

Ammonium Chloride.—Since ammonium chloride solution is occasionally employed as an i. m. or i. v. injection or infusion for the prevention of alkalosis, special mention is made of its hemolytic activity. Figure 1 shows a graph of the degree of hemolysis of erythrocytes in various concentrations of ammonium chloride solutions. Instead of obtaining the characteristic (17) S-shaped curve (sigmoid) as in the case of sodium chloride solutions, one obtains a radically different curve, Fig. 1. As the concentration of ammonium chloride increases, the degree of hemolysis decreases from 93% until it reaches a minimum point between 4 and 5% ammonium chloride concentration. Four per cent ammonium chloride solution was found to require

TABLE I.—PERCENTAGE HEMOLYSIS OF ERYTHROCYTES IN ISO-OSMOTIC SOLUTIONS

Substance	Iso-osmotic Concentration, %	Hemolysis, %	Approx. pH
Acetazoleamide sodium	3.85	0	9.2
Alcohol U. S. P.	1.39	100	6.0
Alcohol, dehydrated N. F.	1.28	100	6.1
Alum (potassium) N. F.	6.35	24[a]	3.4
Amiodoxyl benzoate	4.42	2	4.4
Ammonium carbonate U. S. P.	1.29	97	7.7
Ammonium chloride U. S. P.	0.80	93	5.0
Ammonium lactate	2.76	98	5.9
Ammonium nitrate	1.30	91	5.3
Ammonium phosphate, dibasic	1.76	0	7.9
Ammonium sulfate	1.68	0	5.3
Amobarbital sodium U. S. P.	3.60	0	9.3
d-Amphetamine HCl	2.64	98	5.7
Amphetamine phosphate N. F.	3.47	0	4.5
Amphetamine sulfate U. S. P.	4.23	0	5.9
Amydricaine HCl	5.74	84	6.8
Amylcaine HCl	4.98	100	5.6
Antipyrine N. F.	6.81	100	6.1
2-Methylamino-6-hydroxy-6-methylheptane (Aranthol)	3.96	100[b]	12.0
Arecoline HBr N. F.	3.88	41	4.4
Ascorbic acid U. S. P.	5.04	100[c]	2.2
Atropine sulfate U. S. P.	8.85	0	5.0
Barbital sodium	3.12	0	9.8
Boric acid U. S. P.	1.90	100	4.6
Butethamine formate	4.56	93	6.3
Caffeine and sodium benzoate U. S. P.	3.92	0	7.0
Caffeine and sodium salicylate U. S. P.	5.77	0	6.8
Calcium chloride U. S. P.	1.70	0	5.6
Calcium chloride ($6H_2O$)	2.50	0	5.7
Calcium chloride, anhydrous	1.30	0	5.6
Calcium lactate N. F.	4.50	0	6.7
Calcium pantothenate U. S. P.	5.50	0	7.4
Chloramine-T N. F.	4.10	100[c]	9.1
Citric acid U. S. P.	5.52	100[c]	1.8
Cocaine HCl U. S. P.	6.33	47	4.4
Codeine phosphate U. S. P.	7.29	0	4.4
p-Propylaminobenzoic acid-γ-dimethylamino-β-oxypropyl ester HCl (Cornecaine)	7.30	100	6.0
Cupric sulfate N. F.	6.85	trace[d]	3.9
Cupric sulfate, anhydrous	4.09	trace[d]	4.0
Cyclopentamine HCl	2.68	100	5.7
Decamethonium bromide	5.00	0	5.7
Dextroamphetamine phosphate N. F.	3.60	0	4.7
Dextroamphetamine sulfate U. S. P.	4.20	0	5.9
Dextrose U. S. P.	5.51	0	5.9
Dextrose, anhydrous	5.05	0	6.0
Diethylcarbamazine citrate	6.29	100[c]	3.7
Dihydrostreptomycin sulfate U. S. P.	19.40	0	6.1
Dipyrone	4.65	0	7.3
Edrophonium chloride	3.36	0	4.5
Ephedrine HCl N. F.	3.20	96	5.9
Ephedrine sulfate U. S. P.	4.54	0	5.7
Epinephrine bitartrate U. S. P.	5.70	100[c]	3.4
Ethylmorphine HCl U. S. P.	6.18	38	4.7
Ethylnorepinephrine HCl	3.32	5	4.2
Fluorescein sodium U. S. P.	3.34	0	8.7
d-Fructose	5.05	0[e]	5.9
Galactose	4.92	0	5.9
Glycerin U. S. P.	2.60	100	5.9
Glyphylline	10.87	95	5.9
Guanidine HCl	1.47	0	4.7
Hexamethonium bromide	4.99	0	6.0
Hexamethonium chloride	3.30	0	4.7
Histalog	1.91	100[c]	1.6
Histamine di-HCl	2.24	79[f]	3.7
Homatropine HBr U. S. P.	5.67	92	5.0
Hydroxyamphetamine HBr U. S. P.	3.71	92	5.0
Intracaine HCl	4.97	85	5.0
Iodophthalein sodium U. S. P.	9.58	100	9.4
Isoniazid U. S. P.	4.35	100	7.1
Lactic acid U. S. P.	2.30	100[c]	2.1
Lactose U. S. P.	9.75	0[e]	5.8
Magnesium chloride	2.02	0	6.3
Magnesium sulfate	6.30	0	6.2
Mannitol N. F.	5.07	0[e]	6.2
Menadione sodium bisulfite U. S. P.	5.07	0	5.3
Meperidine HCl U. S. P.	4.80	98	5.0
Mephentermine sulfate U. S. P.	4.74	0	4.5
Methacholine bromide	3.77	0	5.0
Methacholine chloride U. S. P.	3.21	0	4.5
Methadone HCl U. S. P.	8.59	100[c]	5.0
Methamphetamine HCl U. S. P.	2.75	97	5.9
Methenamine U. S. P.	3.68	100	8.4
Methoxamine HCl U. S. P.	3.82	88	5.2
Methylatropine bromide	7.03	0	5.7
Monoethanolamine N. F.	1.76	100	11.4
Naphazoline HCl N. F.	3.99	100	5.3
Neoarsphenamine	2.32	17	7.8
Nicotinamide U. S. P.	4.49	100	7.0
Nikethamide U. S. P.	5.94	100	6.9
Oxophenarsine HCl U. S. P.	3.67	trace[a]	2.3
Penicillin G, potassium U. S. P.	5.48	0	6.2
Pentylenetetrazole U. S. P.	4.91	100	6.7
Phenobarbital sodium U. S. P.	3.95	0	9.2
Phenol U. S. P.	2.80	0[b]	5.6
Phenylephrine HCl U. S. P.	3.00	0	4.5
Phenylpropanolamine HCl	2.60	95	5.3
Phenylpropylmethylamine HCl	2.70	95	5.4
Pilocarpine HCl U. S. P.	4.08	89	4.0
Potassium chlorate N. F.	1.88	0	6.9
Potassium chloride U. S. P.	1.19	0	5.9
Potassium iodide U. S. P.	2.59	0	7.0
Potassium nitrate N. F.	1.62	0	5.9
Potassium phosphate N. F.	2.08	0	8.4
Potassium phosphate, monobasic	2.18	0	4.4
Potassium sulfate	2.11	0	6.6
Probarbital sodium N. F.	3.10	0	10.0
Procaine HCl U. S. P.	5.05	91	5.6

TABLE I (*continued*)

Substance	Iso-osmotic Concentration, %	Hemolysis, %	Approx. pH
Propylene glycol U. S. P.	2.00	100	5.5
Quinine and urea HCl N. F.	4.50	64[f]	2.9
Racephedrine HCl N. F.	3.07	94	5.7
Resorcinol U. S. P.	3.30	96	5.0
Scopolamine HBr U. S. P.	7.85	8	4.8
Silver nitrate U. S. P.	2.74	0[d]	5.0
Silver protein, mild N. F.	5.51	0	9.0
Sodium acetate, anhydrous	1.18	0	8.1
Sodium aminosalicylate U. S. P.	3.27	0	7.3
Sodium arsenate, dibasic	3.83	0	8.8
Sodium ascorbate	3.00	0	6.9
Sodium benzoate U. S. P.	2.25	0	7.5
Sodium bicarbonate	1.39	0	8.3
Sodium biphosphate, anhydrous	2.10	0	4.1
Sodium biphosphate U. S. P.	2.45	0	4.1
Sodium biphosphate ($2H_2O$)	2.77	0	4.0
Sodium bisulfite U. S. P.	1.50	0[c]	3.0
Sodium borate U. S. P.	2.60	0	9.2
Sodium cacodylate N. F.	3.30	0	8.0
Sodium carbonate, anhydrous	1.32	100	11.1
Sodium carbonate, monohydrated U. S. P.	1.56	100	11.1
Sodium chloride U. S. P.	0.90	0	6.7
Sodium citrate U. S. P.	3.02	0	7.8
Sodium iodide U. S. P.	2.37	0	6.9
Sodium lactate	1.72	0	6.5
Sodium metabisulfite	1.38	5[f]	4.5
Sodium nitrate	1.36	0	6.0
Sodium nitrite U. S. P.	1.08	0[a]	8.5
Sodium phosphate, exsiccated, N. F.	1.75	0	9.1
Sodium phosphate N. F.	3.33	0	9.2
Sodium phosphate, dibasic ($2H_2O$)	2.23	0	9.2
Sodium phosphate, dibasic ($12H_2O$)	4.45	0	9.2
Sodium propionate N. F.	1.47	0	7.8
Sodium salicylate U. S. P.	2.53	0	6.7
Sodium sulfate N. F.	3.95	0	6.1
Sodium sulfate, anhydrous	1.61	0	6.2
Sodium thiosulfate N. F.	2.98	0	7.4
Sorbitol ($\frac{1}{2}H_2O$)	5.48	0	5.9
Succinylcholine chloride U. S. P.	4.48	85[a]	3.5
Sucrose U. S. P	9.25	0	6.4
Sulfacetamide sodium U. S. P.	3.85	0	8.7
Sulfadiazine sodium U. S. P.	4.24	0	9.5
Sulfathiazole sodium N. F.	4.82	0	9.9
Tartaric acid N. F.	3.90	75[f]	1.7
Tetraethylammonium bromide	3.17	0	5.7
Tetraethylammonium chloride	2.67	0	4.7
Thiamine HCl U. S. P.	4.24	87[f]	3.0
Tolazoline HCl U. S. P.	3.05	93	4.8
Trimethadione U. S. P.	4.22	100	6.0
Tropacocaine HCl	4.92	75	5.7
Tuaminoheptane sulfate N. F.	3.40	0	5.9
Urea U. S. P.	1.63	100	6.6
Urethan U. S. P.	2.93	100	6.3
Vinbarbital sodium	3.55	0	9.7

[a] R. B. cells turned black color. [b] Solution turned olive-green color. [c] Solution turned brown-black color. [d] R. B. cells shrunk in size and turned black color. [e] R. B. cells clumped. [f] Solution and R. B. cells darkened. [g] Solution became slightly turbid. [h] R. B. cells turned brown color and solution became milky. [i] R. B. cells turned violet color. [j] Solution and R. B. cells turned violet color.

the presence of 2.5% dextrose to prevent hemolysis. The degree of hemolysis is then greater in the ammonium chloride solutions from this minimum point up to 10%.

Various proportions of sodium chloride were added to three different concentrations of ammonium chloride solution: 0.8, 0.6, and 0.4%. It was found that an approximately identical proportion of sodium chloride 0.65%, was required to prevent hemolysis of erythrocytes by each of the ammonium chloride solutions, Table III.

The commonly accepted iso-osmotic composition of 0.8% ammonium chloride gives 93% hemolysis. To prevent hemolysis of erythrocytes, it was found under the conditions of the procedure mentioned previously that 0.65% sodium chloride or 4% dextrose must be added to the 0.8% ammonium chloride solution. This will give an isotonic but hyperosmotic solution.

Amphetamine Compounds.—The hemolysis obtained in solutions of the amphetamine salts of monovalent and polyvalent anions provides an interesting comparison with the ammonium salts. Iso-osmotic solutions of amphetamine hydrochloride, hydrobromide, and methamphetamine hydrochloride each produced about 100% hemolysis while the solutions of amphetamine phosphate and sulfate prevented hemolysis. The erythrocytes are more permeable to the monovalent anions of amphetamine than to the polyvalent sulfate and phosphate anions. This is in agreement with Davson (16) and Thomasson and Husa (7) who found that the sulfate and other polyvalent anions penetrate the erythrocyte very slowly in comparison with univalent anions.

Ephedrine Compounds.—Thomasson and Husa (7) mentioned the difference in the hemolytic activity between ephedrine hydrochloride and sulfate solutions. In the present study racephedrine hydrochloride was also found to allow nearly the same degree of hemolysis as ephedrine hyprochloride 94 and 100%, whereas ephedrine sulfate prevented hemolysis. This is, one would assume, because the membrane is more permeable to the chloride than to the sulfate ion.

Because of the great difference in their hemolytic effects, solutions of ephedrine hydrochloride and sulfate were investigated further in order to find out how much sodium chloride and sodium sulfate must be added to prevent the hemolysis of their iso-

TABLE II.—ERYTHROCYTE HEMOLYSIS IN ISO-OSMOTIC SOLUTIONS OF VARIOUS AMMONIUM SALTS

Substance	Hemolysis, %	Substance	Hemolysis, %
Ammonium acetate[a]	100	Ammonium citrate[a]	0
Ammonium benzoate[a]	100	Ammonium phosphate, dibasic	0
Ammonium carbonate	98	Ammonium sulfate	0
Ammonium chloride	93	Ammonium tartrate[a]	0
Ammonium lactate	98	Tetraethyl-ammonium bromide	0
Ammonium nitrate	92	Tetraethyl-ammonium chloride	0
Ammonium salicylate[a]	100		

[a] Results from Cadwallader and Husa (6).

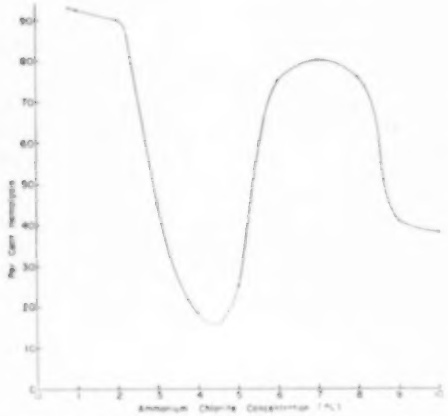

Fig. 1.—Erythrocyte hemolysis in ammonium chloride solutions of varying concentrations.

osmotic solutions and of various dilutions of these. Various proportions of sodium chloride were added to iso-osmotic solutions of ephedrine hydrochloride and the degree of hemolysis was determined, Table IV.

When 0.6% sodium chloride is added, the hemolysis is prevented. This is slightly more than the required amount of sodium chloride which alone would retard hemolysis. Thus the ephedrine hydrochloride is similar to ammonium chloride and other substances, as reported by Thomasson and Husa (7), which act in a negative manner in preventing hemolysis in the presence of sodium chloride. In other words, their iso-osmotic solutions cause an increased fragility of erythrocytes.

The per cent of hemolysis given by various proportions of the iso-osmotic concentration of ephedrine hydrochloride solutions containing the necessary amounts of sodium chloride or of sodium sulfate to maintain iso-osmoticity was determined

TABLE III.—ERYTHROCYTE HEMOLYSIS IN AMMONIUM CHLORIDE SOLUTIONS CONTAINING VARYING AMOUNTS OF SODIUM CHLORIDE

NaCl Added, %	Hemolysis, % — Ammonium Chloride Concentration 0.8%	0.6%	0.4%
0.50	82	80	76
0.55	45	43	39
0.60	3	6	2
0.65	0	0	0
0.70	0	0	0
0.80	0	0	0

TABLE IV.—ERYTHROCYTE HEMOLYSIS IN ISO-OSMOTIC EPHEDRINE HYDROCHLORIDE SOLUTIONS (3.2%) CONTAINING VARYING PROPORTIONS OF SODIUM CHLORIDE

NaCl Added, %	Hemolysis, %
0.3	94
0.4	91
0.5	6
0.6	0
0.7	0

TABLE V.—ERYTHROCYTE HEMOLYSIS IN VARIOUS EPHEDRINE HYDROCHLORIDE SOLUTIONS MADE ISO-OSMOTIC WITH SODIUM CHLORIDE

Ephedrine HCl — Iso-osmotic Multiple	Concn., %	NaCl Added — Iso-osmotic Multiple	Concn., %	Hemolysis, %
1 1/4	4.0	0	0	99
1	3.2	0	0	96
3/4	2.4	1/4	0.23	96
1/2	1.6	1/2	0.45	94
3/8	1.2	5/8	0.56	1
1/4	0.8	3/4	0.68	0

TABLE VI.—ERYTHROCYTE HEMOLYSIS IN VARIOUS EPHEDRINE HYDROCHLORIDE SOLUTIONS MADE ISO-OSMOTIC WITH SODIUM SULFATE

Ephedrine HCl — Iso-osmotic Multiple	Concn., %	Na_2SO_4 Added — Iso-osmotic Multiple	Concn., %	Hemolysis, %
1	3.2	0	0	96
3/4	2.4	1/4	0.38	16
1/2	1.6	1/2	0.76	0
1/4	0.8	3/4	1.14	0

and the results are presented in Table V for the addition of sodium chloride and Table VI for the addition of sodium sulfate.

Ephedrine hydrochloride solutions have strong hemolytic activity until their concentrations are reduced to less than one-half iso-osmotic value with sodium chloride.

The presence of sodium sulfate depresses much more strongly the hemolytic activity of ephedrine hydrochloride than does an equivalent quantity of sodium chloride.

The per cent of hemolysis given by various proportions of the iso-osmotic concentration of ephedrine sulfate solutions adjusted with sodium chlo-

TABLE VII.—ERYTHROCYTE HEMOLYSIS IN EPHEDRINE SULFATE SOLUTIONS MADE ISO-OSMOTIC WITH SODIUM CHLORIDE

Ephedrine Sulfate		—NaCl Added—		
Iso-osmotic Multiple	Concn., %	Iso-osmotic Multiple	Concn., %	Hemolysis, %
1	4.54	0	0	0
3/4	3.41	1/4	0.23	0
1/2	2.27	1/2	0.45	0
1/4	1.14	3/4	0.68	0

TABLE VIII.—ERYTHROCYTE HEMOLYSIS IN ISO-OSMOTIC BORATE:BORIC ACID SOLUTIONS

Boric Acid Solution		Sodium Borate Solution			
Volume, ml.	Mole Fraction	Volume, ml.	Mole-Fraction	Hemolysis, %	pH
50	1.00	0	0	100	4.7
45	0.98	5	0.02	99	7.1
40	0.95	10	0.05	99	7.6
35	0.91	15	0.09	99	7.9
30	0.87	20	0.13	97	8.1
25	0.82	25	0.18	23	8.4
20	0.75	30	0.25	1	8.6
15	0.66	35	0.34	0	8.8
10	0.53	40	0.47	0	8.9
5	0.34	45	0.66	0	9.0
0	0	50	1.00	0	9.2

ride was determined and the results are given in Table VII.

Ephedrine sulfate solutions prevented hemolysis of red blood cells even though there was an appreciable quantity of chloride present for cellular exchange and for maintenance of cellular neutrality.

Ethanol.—An iso-osmotic (1.39%) solution of ethanol failed completely to prevent hemolysis of erythrocytes. It was found necessary to add 0.5% sodium chloride to the ethanol solution to prevent hemolysis. This is the same amount of sodium chloride which alone will just prevent hemolysis. The ethanol in iso-osmotic concentration, therefore, did not increase the fragility of the erythrocytes as did the ammonium chloride.

Erythrocyte Hemolysis in the System: Boric Acid-Sodium Borate.—Since an iso-osmotic solution (1.9%) of boric acid fails to prevent hemolysis of erythrocytes while an iso-osmotic solution (2.6%) of sodium borate prevents hemolysis, a study was made to show the degree of erythrocyte hemolysis in various proportions of the two iso-osmotic solutions, Table VIII. The pH of each solution was obtained before the addition of the blood sample. For comparative purposes the quantities of the two solutes in the various mixtures are reported as mole fractions of the total solute only.

Since boric acid is more ether soluble than is sodium borate, it is undoubtedly more lipoid soluble, and therefore it penetrates the plasma membrane of the erythrocyte more readily than does the sodium borate. The concentration of the reactants in the mixture which prevented hemolysis was 0.75 or less mole-fractions of boric acid and 0.25 or more mole-fractions of sodium borate, and with a pH 8.6 to 9.2. Since an appreciable change in the degree of hemolysis took place in a rather limited pH interval, it can be concluded perhaps that the pH is of no real influence in this instance.

Trolle-Lassen (18) has shown previously that red blood cells hemolyze only to a very slight extent in isotonic solutions of sodium chloride containing 2% or less of boric acid. But in all cases the erythrocytes were completely hemolyzed within a few hours if the solutions contained 3% or more of boric acid.

Reversed Proportion of Erythrocytes to Total Solution Volume.—The results in Table I show that there is a large number of substances which in iso-osmotic concentration fail to prevent hemolysis of red blood cells when a small amount of blood is mixed with a large volume of solution; however, this proportion of solution to blood and tissue fluids (10 ml. solution to 0.1 ml. blood or 100:1) is not very often obtained in the body and hence the danger from hemolysis usually is much less than is indicated by *in vitro* studies. The average proportional volume of a medicinal injection usually is too small when compared to the total blood volume and adjacent tissue fluid volume to be a serious factor in all but exceptional cases.

The hemolytic activities of a few strongly hemolytic substances were determined in the completely reversed proportion of solution to blood volume (1:10). One-half milliliter of iso-osmotic concentration of ammonium chloride, boric acid, ethanol sodium carbonate, and urea was added to 10 ml. of a 1:1 mixture of fresh, defibrinated whole blood and 0.9% sodium chloride solution. The blood was diluted with an equal volume of sodium chloride solution for ease in centrifuging and colorimetric measuring. Blanks were made containing the blood with normal saline solution replacing the hemolytic solution. The incubation and photoelectric determination of the color of the solution was made in a similar manner as before and showed that there was no hemolysis (0%) of the erythrocytes when mixed in proportion of 10:1 with each of the solutions tested.

When the volume of urea solution was increased to 1 ml. (which resulted in a mixture of one part of iso-osmotic urea solution to five parts of blood), there was slight hemolysis (10%). When 2 ml. of urea solution (1 part iso-osmotic urea solution to 2 1/2 parts blood) was investigated, a moderate degree of hemolysis resulted (40%). This indicates that one of the factors in the consideration of the use of isotonic solutions *versus* iso-osmotic solutions is clearly that of the quantity and proportions of the substances used and the resulting rapidity and ease of the dilution of the medicinal solution by the body fluids.

SUMMARY

1. The degree of hemolysis of human erythrocytes was determined in 161 different pharmaceutically employed iso-osmotic solutions.

2. Ninety substances prevented hemolysis in iso-osmotic concentration and 71 substances showed varying amounts of hemolysis.

3. Solutions of carbohydrates, most alkali

salts of organic acids, and inorganic salts with moderate pH prevented hemolysis.

4. Most inorganic and organic acids and alkalies with more extreme pH values or which have marked oxidation or reduction properties usually penetrated or altered the erythrocyte sufficiently to give some degree of hemolysis.

5. Many amine salts usually of a monovalent anion failed to prevent hemolysis, whereas amine salts of di- and tri-valent anions usually did prevent hemolysis.

6. A hemolytic curve for ammonium chloride solution is presented.

7. Special comparisons are made of the hemolytic activity of iso-osmotic solutions of ammonium, amphetamine, and ephedrine compounds. Many of these caused an increased fragility of erythrocytes.

8. An iso-osmotic solution of ethanol requires the addition of 0.5 per cent sodium chloride to prevent hemolysis.

9. In the boric acid-sodium borate system, 0.75 or less mole-fractions of boric acid and 0.25 or more mole-fractions of sodium borate are required to prevent hemolysis.

10. When the usual proportions of iso-osmotic solution to blood is reversed for a few strongly hemolytic substances, much less hemolysis occurs than did previously.

11. There is no distinct pattern indicating the predominance of any single main mechanism of penetration or alteration of the erythrocytes by the solute and solvent which results in hemolysis of the erythrocyte.

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Stability of Morphine in Aqueous Solution II

Separation of Morphine from Its Degradation Products

By SHU-YUAN YEH and JOHN L. LACH

The chromatographic procedure previously described for the separation of morphine from its degradation products has been modified since this procedure was not entirely satisfactory, especially in highly oxidized morphine solutions. In this modified procedure, these higher oxidation products are first removed from the column using 5 per cent *n*-butanol in chloroform. Morphine is then eluted using 15 per cent *n*-butanol in chloroform. Paper chromatographic studies indicate that the decomposition of morphine is extremely complex.

Before a kinetic study of morphine in aqueous solution could be undertaken, an analytical procedure for the separation of the intact morphine molecules from its degradation products was required. Chromatographic procedures for such a separation have been reported by Liang (1), Ramabhadram (2), and Lach, *et al.* (3). An investigation of the procedure of Liang was found to be unsatisfactory in that an excess of eluent was required. The procedure of Ramabhadram was also unsatisfactory since it necessitated an evaporation of the solutions under study to dryness prior to analysis by adsorption chromatography. This could lead to an additional decomposition of morphine. It was found, however, that with certain modifications of the procedure of Lach (3), a quantitative separation of morphine from its degradation

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products could be achieved. This investigation was concerned with the determination of the optimum conditions of pH, polarity of eluent, and amount of supporting medium necessary for quantitative separation. In this study morphine was assumed to undergo oxidation to pseudomorphine and to morphine N-oxide. It was also assumed that these products underwent further degradations.

EXPERIMENTAL

Preparation of Pseudomorphine Sulfate.—Pseudomorphine was synthesized according to the method of Fulton (4), and purified according to the procedure of Balls (5). The purified pseudomorphine base melted with decomposition near 325°.

The sulfate salt was prepared by dissolving the purified base in warm glacial acetic acid and precipitating the sulfate salt by addition of concentrated sulfuric acid. The precipitated pseudomorphine sulfate was recrystallized from hot water and dried under vacuum for six hours.

Preparation of Morphine N-Oxide.—Morphine N-oxide was prepared according to the procedure of Freund and Speyer (6), and recrystallized from alcoholic aqueous solution. Small white prism-like crystals were obtained which melted, with decomposition, at 273°. The nitrate salt of morphine N-oxide melted at 206° which is in agreement with the reported value (6).

Preparation of Degraded Pseudomorphine Sulfate Solution.—Fifty-milliliter ampuls, each containing 25 ml. of 0.1% pseudomorphine sulfate solution, were saturated with oxygen, sealed, and autoclaved for two hours at 15 pounds pressure. These were then placed in an oven at 95° for one week. They were then opened, again saturated with oxygen, and autoclaved for an additional two hours at 15 pounds pressure. The resulting solution was highly colored and contained some precipitated material.

Preparation of Degraded Morphine N-Oxide Solution.—Morphine N-oxide, 25 ml. of 0.1% solution, was transferred to 100-ml. vaccine bottles, saturated with oxygen, stoppered, sealed with an aluminum cap, and placed in a 95° oven. These solutions were removed from the oven after one day, chilled, opened, saturated with oxygen for two minutes, and again sealed and stored in the oven. The process was repeated for one week. The resulting solutions were colored and contained some precipitated material.

Effect of Various Eluents on the Chromatographic Elution of Pseudomorphine Sulfate and Oxidized Pseudomorphine Sulfate.—The procedure of Lach, *et al.* (3), was employed in this study. Fifteen grams of Celite 535,[1] previously washed with chloroform-butanol mixture to remove interfering impurities, was thoroughly mixed with 15 ml. of 1 *M* phosphate buffer pH 6.5, in a 250-ml. beaker. The solvent system under investigation, (3, 5, 15, and 20% *n*-butanol in chloroform, 3 and 5% ethanol in chloroform; chloroform; and benzene) was added to make a slurry. The slurry was then transferred and packed in a chromatographic column.

The samples were prepared by pipetting 3 ml. of pseudomorphine sulfate or degraded pseudomorphine sulfate solution and 2 ml. of 1 *M* phosphate buffer at pH 6.5 to 5 Gm. of washed Celite in a 100-ml. beaker. The mixture was triturated, eluent was added, and the slurry quantitatively transferred to the column with frequent packing. The column was then eluted at a flow rate of approximately 2 ml. per minute. The effluent was collected in 10-ml. fractions, and the absorbance of each fraction determined at 286 mμ. Results obtained in this study showed that pure pseudomorphine sulfate is not eluted from the column using 15% *n*-butanol in chloroform as the eluent. The degradation products of pseudomorphine sulfate are, however, removed within a 100-ml. column using *n*-butanol in chloroform in which the concentration of *n*-butanol was as low as 5%. Separation of the degradation products of pseudomorphine sulfate from pure pseudomorphine sulfate itself can thus be achieved by first eluting the column with 5% *n*-butanol in chloroform which removes these degradation products.

The use of eluents such as chloroform and benzene for the removal of the degradation products of pseudomorphine sulfate were unsatisfactory in that only small amounts of these products were removed. The addition of 3 or 5% ethanol in chloroform, and the use of 20% *n*-butanol in chloroform resulted in removal of the water of the internal phase.

Effect of pH on the Chromatographic Elution of Morphine Sulfate, Pseudomorphine Sulfate, and Oxidized Pseudomorphine Sulfate.—An investigation of the effect of pH of the internal phase on the rate of elution of morphine was carried out. Results of this study indicated that, although an alkaline pH was more favorable for the elution of pure morphine, satisfactory separation of the degradation products of pseudomorphine was not achieved using 5% *n*-butanol in chloroform to first remove these bodies. Data also indicated that for maximum separation of morphine from the higher degradation products of pseudomorphine using this initial 5% *n*-butanol in chloroform eluent, the pH of the internal phase was required to be 6.4 to 6.7. At a pH lower than 6.4, recovery of morphine from the column was slow.

Effect of the Quantity of Support on the Chromatographic Elution of Morphine Sulfate and Degraded Pseudomorphine Sulfate.—Since a volume of approximately 100 ml. of eluent was required to elute completely the degradation products of pseudomorphine sulfate at pH 6.5, using 5% *n*-butanol in chloroform as the eluent, a study of the effect of the quantity of Celite used as the supporting medium was undertaken in that trace amounts of pure morphine may also be eluted within this large volume. Elution of the degradation products of pseudomorphine sulfate within a relatively small volume would eliminate this possibility. In this study 10, 15, and 20 Gm. of Celite were employed. This amount of support used included the 5 Gm. of Celite employed in the quantitative transfer of the aqueous sample to the column. The columns were prepared in a manner previously described, and the internal phase con-

[1] Johns-Manville Co.

sisted of a phosphate buffer at pH 6.5. Results of this study, using 5% *n*-butanol in chloroform as the eluent, showed that the use of 10 and 20-Gm. Celite columns gave incomplete separation of the degraded pseudomorphine from morphine (Fig. 1). Satisfactory results were obtained using a total 15-Gm. Celite column. Complete separation of the degradation products was achieved in 60 ml. of effluent, pure morphine being eluted in the eighth 10-ml. fraction.

Chromatographic Separation of Morphine N-Oxide and Its Degradation Products.—The modified chromatographic procedure developed thus far, employing a total 15-Gm. Celite column and an internal phase of pH 6.5 and satisfactory for the separation of morphine from pseudomorphine and its degradation products, was next applied to the separation of morphine N-oxide and its degradation products since morphine N-oxide has also been reported (7) to be one of the degradation products of morphine. Results of this study also showed that pure morphine N-oxide is not eluted from the column using 5% *n*-butanol in chloroform as the eluent, whereas the degradation products were eluted with a volume of 60 ml. of effluent, as in the case of degraded pseudomorphine sulfate. Pure morphine N-oxide was eluted, however, in the seventeenth 10-ml. fraction using 15% *n*-butanol in chloroform as the eluent (Fig. 2). The appearance of pure morphine N-oxide in the seventeenth 10-ml. fraction does not interfere with the separation of pure morphine since morphine is collected from the seventh through the sixteenth fraction, representing a total volume of 100 ml., using 15% *n*-butanol in chloroform as the eluent.

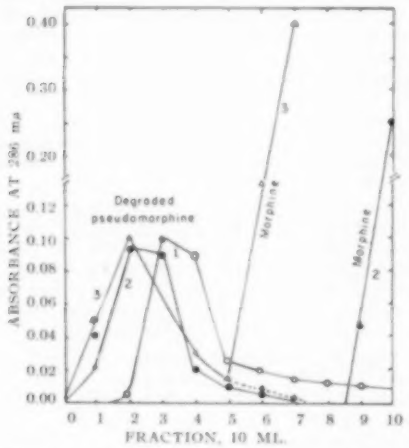

Fig. 1.—Effect of quantity of support on elution of morphine and its degraded products, using 5% *n*-BuOH in $CHCl_3$ as eluent: curve 1, 20 Gm.; curve 2, 15 Gm.; curve 3, 10 Gm. Celite.

ANALYTICAL CHROMATOGRAPHIC PROCEDURE

The procedure as described below was used throughout the course of the kinetic investigation.

The chromatographic columns were prepared by thoroughly mixing (triturating) 10 Gm. of Celite with 10 ml. of 1 *M* phosphate buffer at pH 6.5, in a 250-ml. beaker. A slurry was prepared by adding 50 ml. of a 5% *n*-butanol in chloroform to the mixture. The slurry was then transferred to a column (2 cm. × 45 cm.) and packed in the usual manner.

Exactly 3 ml. of the decomposed morphine sulfate solution sample was pipetted onto 5 Gm. of Celite contained in a 100-ml. beaker. To this was added a sufficient amount of 1 *M* dibasic phosphate solution, previously determined, to bring the sample to pH 6.5, and a sufficient amount of 1 *M* phosphate buffer at pH 6.5, to bring the total volume to 5 ml. The addition of 1 *M* dibasic phosphate solution to

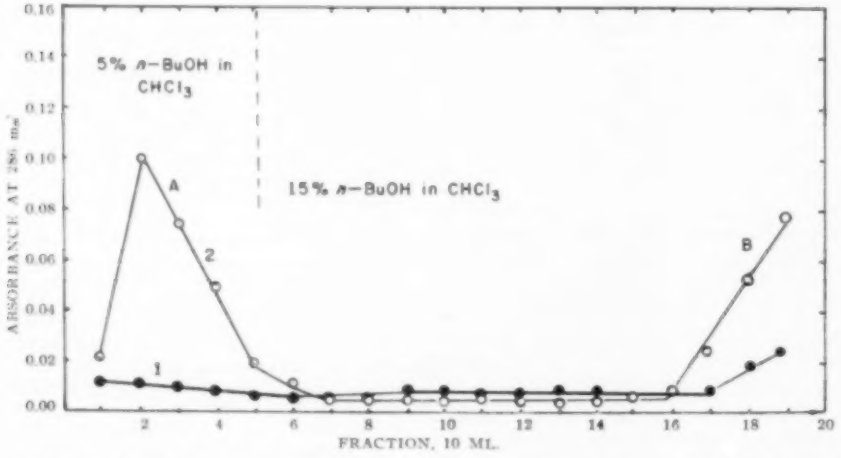

Fig. 2.—Elution of morphine N-oxide and degraded morphine N-oxide. Curve 1: 3ml. (3 mg.) morphine N-oxide. Curve 2: 1 ml. (1 mg.) degraded morphine N-oxide.

the 3-ml. morphine sulfate sample prior to the preparation of the slurry was necessary in that it was essential that the final pH of the internal phase be 6.5 for complete separation of morphine from its degradation products. A pH beyond 6.4 to 6.7 gave incomplete separation. This mixture was thoroughly triturated. Approximately 25 ml. of 5% *n*-butanol in chloroform was added to make a slurry which was quantitatively transferred to the column and packed in the usual manner. A plug of glass wool was placed on the top of the packed column. The beaker and the glass plunger were rinsed with 10 ml. of 5% *n*-butanol in chloroform. The flow rate was adjusted to approximately 2 ml. per minute. When 50 ml. of effluent was collected the eluent was changed to 15% *n*-butanol in chloroform, and an additional 10 ml. of effluent was collected. This 60-ml. portion of effluent containing the higher degradation products of morphine was discarded. The intact morphine was then obtained by collecting exactly 100 ml. of effluent in a volumetric flask and its concentration determined by measuring the absorbence at 286 mμ, using a blank which was obtained by passing the butanol-chloroform solvent through a Celite column.

In the procedure the Celite was not washed with chloroform-butanol prior to use since the first 60-ml. fraction of 5% *n*-butanol in chloroform removes any impurities and is discarded.

PAPER CHROMATOGRAPHY

Paper chromatographic procedures have been applied to the separation of morphine and pseudomorphine by Hosoya and Brody (8), Douhairie (9), and Markavie, *et al.* (10). Hosoya and Brody, using a solvent mixture of *n*-butanol-acetic acid-water (40:10:20) reported an R_f value of 0.69 for morphine and 0.31 for pseudomorphine. Since, in our modified chromatographic procedure, morphine was assumed to decompose to pseudomorphine and morphine N-oxide, and that these products were assumed to undergo further decomposition, it was felt that paper chromatographic techniques would aid in identification of some of these breakdown products.

Suitable amounts of pure and degraded aqueous solutions of morphine, pseudomorphine, and morphine N-oxide were placed on 18 × 22½ inch sheets of Whatman No. 1 chromatographic filter paper. Descending chromatograms were developed for twenty to twenty-two hours at room temperature in a solvent mixture of *n*-butanol (40 vol.), glacial acetic acid (10 vol.), and water (20 vol.). All chromatograms were placed in a Chromatocab previously equilibrated with the solvent mixture for twenty-four hours. The separation of the compounds on the chromatograms was visualized under ultraviolet light. Data representing the average of 5 determinations are reported in Fig. 3. The R_f values, calculated from the original to the center of the spot, for morphine, pseudomorphine, and morphine N-oxide were 0.65, 0.36, and 0.69, respectively, which is in agreement with that reported for morphine and pseudomorphine. The data obtained in this study point out the complexity of the degradation of morphine. Although no distinct spot was observed for the presence of pseudomorphine in the chromatograms for degraded morphine solutions, it should be pointed out that these solutions were highly oxidized and it is quite likely that any pseudomorphine present would have also decomposed under the extreme conditions used for the degradation of the morphine solutions. It has also been reported that pseudomorphine is unstable (5). No attempt was made to identify any of the degradation products of pseudomorphine or morphine N-oxide.

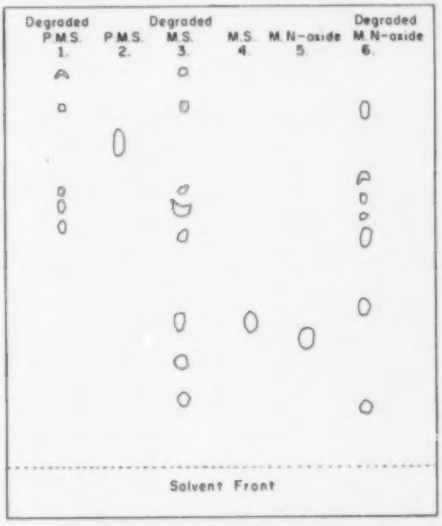

Fig. 3.—Reproduction of chromatogram demonstrating separation of degradation products of morphine sulfate: position 1, 20 μl. of 0.1% degraded pseudomorphine sulfate; 2, 10 μl. of 0.1% pseudomorphine sulfate; 3, 50 μl. of 0.3% degraded morphine sulfate; 4, 10 μl. of morphine sulfate; 5, 10 μl. of 0.1% morphine N-oxide; 6, 20 μl. of 0.1% degraded morphine N-oxide.

Investigation of descending chromatograms using buffered filter paper (1 *M* phosphate buffer, pH 6.5) and 5% and 15% *n*-butanol in chloroform as the solvent were unsuccessful in that no separation or migration of the applied solutions was observed.

CONCLUSION

From data obtained in this study of the effect of pH, eluents, and quantity of support used in this chromatographic procedure, it was concluded that morphine could be satisfactorily separated from pseudomorphine, morphine N-oxide, and their degradation products using a total of 15 Gm. of Celite as the support, an internal phase pH 6.5, 5 per cent *n*-butanol in chloroform for elution of these higher oxidized products within 60 ml., and 15 per cent *n*-butanol in chloroform for the elution of pure morphine within 100 ml. The success of this

TABLE I.—SEPARATION AND RECOVERY OF MORPHINE SULFATE FROM PSEUDOMORPHINE SULFATE ON CELITE COLUMN

Sample Added	Absorbance at 286 mμ, 100-ml. Fraction	Absorbance at 286 mμ, 10-ml. Fraction after 100 ml. Collected	Recovery, %
8.006 mg. M_2SO_4[a] 2 mg. PMS[b]	0.348	0.003	100.8
8.006 mg. M_2SO_4	0.345	0.004	100.0
	0.347 (Diluted to 100 ml. with H_2O)[c]		
3 ml. Degraded PMS	0.007		
8.006 mg. M_2SO_4 2 ml. Degraded PMS	0.347	0.000	100.5
3 ml. Degraded PMS	0.006		
12.009 mg. M_2SO_4 1 ml. Degraded PMS	0.517	0.003	99.9
12.009 mg. M_2SO_4	0.517	0.004	99.9
16.012 mg. M_2SO_4	0.680	0.000	100.0
20.006 mg. M_2SO_4	0.840	0.004	100.0
30.024 mg. M_2SO_4	1.280	0.027	95.8

[a] M_2SO_4 = morphine sulfate. [b] PMS = Pseudomorphine sulfate. [c] Morphine sample assayed spectrophotometrically directly.

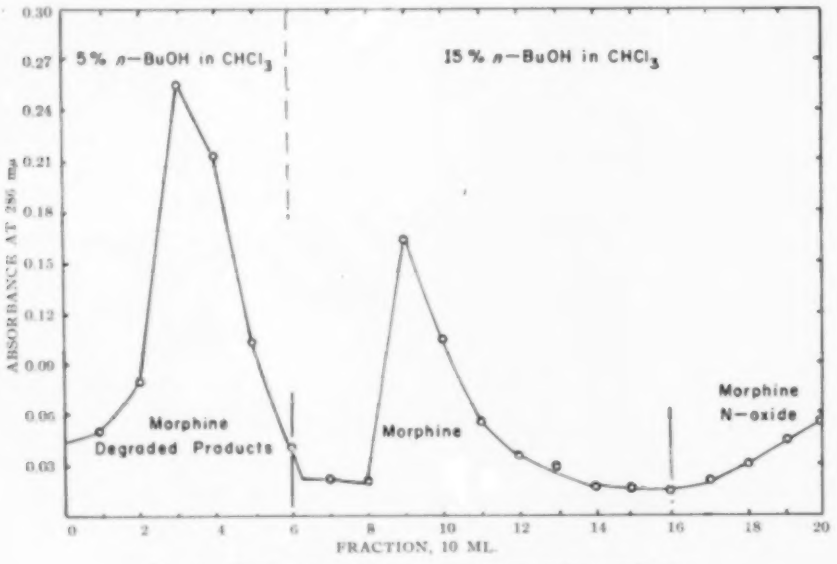

Fig. 4.—Elution of morphine from its degradation products.

method is illustrated in Table I. Quantitative recovery of morphine was obtained when the concentration of pure morphine sulfate did not exceed 25 mg. per 3-ml. sample size. Figure 4 shows a chromatogram obtained using a sample of morphine sulfate in 0.2 *M* phosphate buffer at pH 6.5 which had been stored at 95° and saturated with oxygen daily for two weeks.

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Stability of Morphine in Aqueous Solution III

Kinetics of Morphine Degradation in Aqueous Solution

By SHU-YUAN YEH and JOHN L. LACH

The degradation of morphine in aqueous solution is dependent on the pH of the solution and on the presence of atmospheric oxygen in the system. The overall reaction rate, in systems containing excess oxygen, was found to be equal to

$$\left[k_1' \left(\frac{Ka}{Ka + H^+}\right) + k_2' \left(\frac{H^+}{Ka + H^+}\right)\right] (\text{Morphine})$$

Degradation mechanisms for morphine, based on kinetic data and previously reported data on naphthol oxidation, are presented.

MORPHINE has been used as an analgesic and sedative since its isolation in 1805. Due to the limited solubility of morphine base, the acid salts, chiefly as the sulfate and hydrochloride, have been used extensively in various pharmaceutical preparations.

The stability of morphine in aqueous solution has been studied by many investigators since such solutions, after prolonged storage, undergo decomposition, as evidenced by discoloration. This decomposition of morphine is believed to be due to an oxidation reaction resulting in the formation of pseudomorphine (oxymorphine) and morphine N-oxide in the ratio of 9:1, together with a trace of a base believed to be methylamine (1). The oxidation of morphine and subsequent condensation to the dimer pseudomorphine is assumed to involve the phenolic group, as in the oxidation of naphthols to dimolecular compounds. Morphine derivatives not possessing the free phenolic group, as in the case of codeine and diacetyl morphine, do not undergo this type reaction (2). The oxidation of morphine is catalyzed by oxygen of air (3), sunlight (4), ultraviolet irradiation (5), iron and organic impurities (6), rat liver slices (7), tissue homogenates (8), and cytochrome (9). Ionescu-Matin, *et al.* (10), claimed that the deterioration of morphine in presence of oxygen takes place through condensation of morphine at the phenolic hydroxy group with the formation of the dimer, pseudomorphine. However, in the absence of oxygen, and in the presence of light, they also claimed that the deterioration is due to peroxidation or dimerization which can take place through the oxygen of the hydroxy group which was thought to be activated by ultraviolet light, resulting in the formation of bimorphine. Abood and Kun (8) reported that in the course of oxidation of morphine by tissue, one mole of morphine utilizes one-half mole of oxygen and that the phenolic hydroxy group is oxidized to a quinone. Thorn and Agren (11) reported that the pseudomorphine formed in this oxidation was extremely stable and did not undergo further decomposition. However, Balls (12) pointed out that pseudomorphine was quite unstable, that it decomposes on either oxidation or reduction, and that in alkaline solution pseudomorphine gradually decomposed to higher oxidized products.

The stability of morphine in aqueous solution is largely dependent on the hydrogen ion concentration. In alkaline or neutral solution, morphine deteriorates rapidly at room temperature, whereas acidic solutions are relatively stable (11, 13, 14). The effect of temperature on the stability of morphine has been reported to be less important than the hydrogen ion concentration (14).

Although the stability of morphine in aqueous solution has been extensively investigated, no quantitative studies have been conducted. This report deals with a kinetic study of the degradation of morphine in aqueous solution, the degradation of these solutions carried out in a light proof oven. No study was made to investigate the effect of light on morphine degradation.

EXPERIMENTAL

Reagents and Apparatus

Morphine sulfate U. S. P. recrystallized from alcoholic aqueous solution and dried under vacuum for six hours, m. p. 250°; Beckman spectrophotometer, model DU, equipped with photomultiplier, 1-cm. silica cells. The buffers used in this study are: acetate buffer: 0.2 *M* and 0.4 *M* at pH 3.0, 4.5, 5.0, and 5.5; phosphate buffer: 0.2 *M* and 0.4 *M* at pH 4.0, 5.0, 5.5, 6.0, 6.5, and 7.0. Phosphate

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buffer, 0.2 *M*, at pH 2.0 and 2.5 were made by adjusting 0.1933 *M* phosphoric acid with monopotassium phosphate. All buffer solutions were adjusted using a Beckman pH meter model H2. The phosphate buffer solutions used here are independent of the temperature (15), and the change of pH of acetate buffer at higher temperature is negligible (16).

Degradation of Morphine Sulfate in Sealed Ampuls

Effect of pH on the Degradation of Morphine Sulfate Solution Sealed Under Atmosphere at 95°.—Five milliliters of 0.3% morphine sulfate solution in 0.2 *M* phosphate buffer; pH 2.0, 6.0, 6.5, and 7.0, was introduced into 5-ml. ampuls, sealed under atmosphere, and stored in an oven at 95°. These solutions were assayed for morphine content at various time intervals by a previously described chromatographic procedure (17).

The results of the study are shown in Fig. 1. The data obtained indicate that the rate and extent of decomposition of morphine is dependent on the pH of the solution. It is also apparent that after a time interval, this decomposition is halted, as evidenced by the plateaus. This behavior was probably due to the lack of oxygen in the systems. Calculation of the atmospheric oxygen present in the solutions contained in the ampuls (including the void space) was approximately 3.2×10^{-3} *M*/L. Since at pH 7.0 approximately 3.1×10^{-4} *M*/L. of morphine had undergone decomposition, it appeared that morphine and oxygen react on a mole to mole basis.

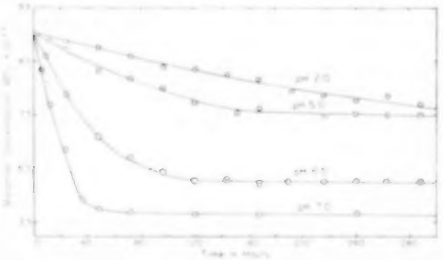


Fig. 1.—Effect of pH on degradation of morphine sulfate in 0.2 *M* phosphate buffer, sealed under atmosphere, at 95°.

In order to verify the oxygen dependency of the reaction, the following study was undertaken. Five milliliters of 0.3% morphine sulfate solution was introduced into 5-ml. ampuls and sealed under sulfur dioxide, atmosphere, commercial nitrogen, and absolute nitrogen. An additional solution of morphine sulfate containing 1% of sodium bisulfite was prepared and sealed under atmosphere. Using sulfur dioxide and commercial nitrogen, the ampuls were bubbled for one minute, three minutes for absolute nitrogen, prior to sealing. These ampuls were stored in a 95° oven and assayed for morphine at periodic time intervals. Results of this study are shown in Figs. 2 and 3. The data indicated that the rate of degradation of morphine is oxygen-dependent since solutions sealed under absolute

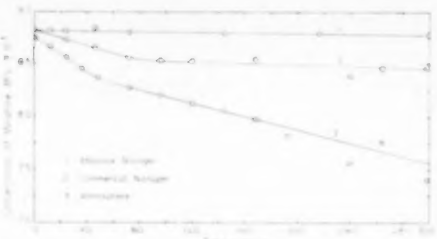


Fig. 2.—Effect of inner gas on degradation of morphine sulfate in deionized water, at 95°.

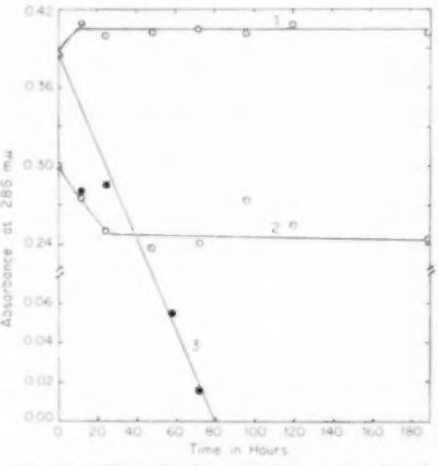


Fig. 3.—Effect of sodium bisulfite, sulfur dioxide on degradation of morphine sulfate at 95°: 1 and 2 contained 1% $NaHSO_3$, sealed under atmosphere; 3, M_2SO_4 in deionized H_2O, sealed under SO_2; 1, assayed by direct spectrophotometric method; 2 and 3, by the chromatographic method.

nitrogen showed no evidence of decomposition. The slight degradation noticed in systems saturated with commercial nitrogen was probably due to impurities in the nitrogen. Absolute nitrogen was prepared by passing commercial nitrogen through three wash bottles containing Fieser's solution (18) and through a saturated solution of lead acetate.

It is interesting to point out here that morphine solutions underwent a color change to yellow when sulfur dioxide was introduced. This color intensity was dependent on the length of time used to saturate the solution with sulfur dioxide. Chromatographic analysis of these freshly prepared sulfur dioxide-saturated solutions gave low recoveries for morphine. Direct spectrophotometric analysis, however, of these same samples, using deionized water as the diluent, gave only slight absorbance difference when compared to the original solutions indicating that some reaction had probably taken place. These sulfur dioxide solutions become highly colored after three to four days' storage at 95° and developed a yellow precipitate after one month. An investigation of this phenomenon is, at present, continuing.

It was also noted that the chromatographic

analysis of the morphine sulfate solution containing 1% sodium bisulfite prior to sealing and storage at 95° resulted in low recovery of morphine (70%). Direct spectrophotometric analysis of the freshly prepared solution, using deionized water as the diluent gave, on the other hand, higher values for morphine. Further study dealing with this aspect showed there was no appreciable change in pH of the solution, although the peak of the spectrogram was shifted slightly to the lower wavelength from 286 to 284 mμ and a stronger absorbance was noted, indicating that some reaction had taken place between morphine and sodium bisulfite. This phenomenon will be discussed in a future communication (19).

This initial investigation confirmed previous studies and showed that the decomposition of morphine sulfate in aqueous solution was dependent on the hydrogen ion and oxygen concentration of the system. Attempts to employ a manometric procedure using a Warburg manometer in the study of the oxygen dependency regarding the rate of degradation of morphine were unsatisfactory in that morphine sulfate, in 0.2 *M* phosphate buffer pH 7.0 at 60°, underwent only slight color change after twelve hours, the amount of oxygen uptake being negligible. However, it was felt that valuable information with respect to the kinetics of this reaction could be obtained by maintaining a relatively constant oxygen concentration in the system. To this end, the rate of degradation of morphine was studied as a function of hydrogen ion concentration, molarity of buffers used, ionic strength, and concentration of morphine in oxygen-saturated systems.

Degradation of Morphine Sulfate in the Presence of Excess Oxygen

Effect of pH.—Approximately 0.15-Gm. portions of anhydrous morphine sulfate were accurately weighed, transferred to 100-ml. vaccine bottles, and dissolved in 50 ml. of the following buffers: 0.2 *M* phosphate buffer, pH 2.5, 6.0, 6.5, and 7.0; 0.2 *M* acetate buffer, pH 4.0, 4.5, 5.0, and 5.5. Three milliliters of each solution was withdrawn and assayed chromatographically for the original morphine concentration. These solutions were bubbled with oxygen for two minutes, rubber stoppered, sealed with aluminum caps, and placed in a 95° oven. At various time intervals the bottles were removed, chilled, and 3-ml. aliquots of solution were withdrawn. After each sample removal, the remaining solution was again saturated with oxygen for two minutes, and stored in the oven. The process was repeated for a total of ninety-six hours. The data obtained, as shown in Fig. 4, indicated that the rate of degradation of morphine was hydrogen ion-dependent. A plot of the log of the concentration of undecomposed morphine against time gives a straight line, indicating that the reaction is pseudo first order with respect to morphine at constant hydrogen ion and oxygen concentration. A plot of the log of the specific rate constant of these reactions as a function of pH resulted in an "S"-shaped curve (Fig. 5). This plot resembles a dissociation curve and indicates that the rate of degradation of morphine is dependent on the type of morphine species present in the solution. Data obtained from studies conducted in 0.2 *M* phosphate buffer at pH 3.0, 4.0, 4.5, 5.0, 5.5, and 0.4 *M* phosphate buffer at pH 5.0 were unsatisfactory in that the capacity of these buffers was inadequate.

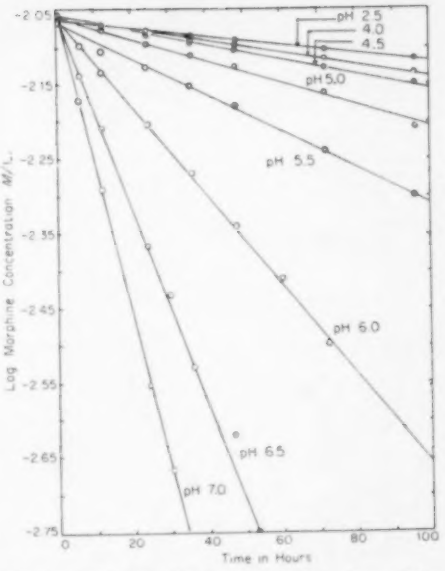


Fig. 4.—Effect of pHs on degradation of morphine sulfate in excess oxygen, at 95°.

Effect of Buffer Molarity and Ionic Strength.—Data obtained in this study using 0.2 *M* and 0.4 *M* acetate buffer at pH 5.0 and 0.2 *M* acetate buffer at pH 5 containing 1 and 3% sodium sulfate indicate that the rate of degradation of morphine is independent of the molarity of buffer and of the ionic strength present, as shown in Figs. 6 and 7.

Effect of Morphine Concentration.—Although data already showed that the overall rate of degradation of morphine at constant hydrogen ion and oxygen concentration is pseudo first order with respect to the concentration of morphine present, this first-order reaction was further verified by a study of 0.3 and 0.15% morphine sulfate in 0.2 *M* acetate buffer, pH 5.0, as shown in Fig. 8.

Effect of Temperature.—Solutions of morphine sulfate in 0.2 *M* phosphate buffer, pH 6.0, 6.5, 0.2 *M* acetate buffer, pH 5.0, were subjected to degradation at 85, 90, and 95°. Results of this investigation are shown in Figs. 9, 10, and 11. By plotting the log of specific rate constant, k, against the reciprocal of temperature, T, as shown in Fig. 12, the apparent energy of activation, E_a, under the condition employed in this study, was calculated from the slopes of these lines and was found to be of the order of 22.8 Kcal.

DISCUSSION

Oxidation of Morphine.—As has already been pointed out, morphine undergoes decomposition resulting in the discoloration of the solution and the formation of precipitates. Our present study indicates that this degradation of morphine is chiefly dependent on the pH of the solution and the pres-

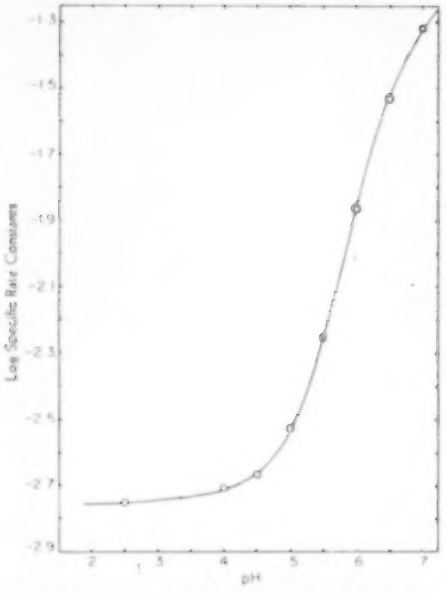


Fig. 5.—Plot of the log of specific rate constants as a function of pH.

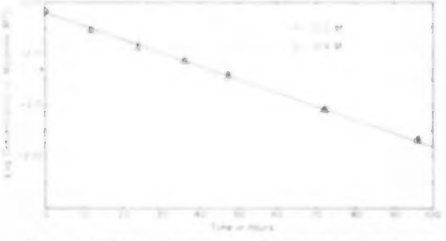


Fig. 6.—Effect of molarity of acetate buffer at pH 5 on degradation of morphine sulfate, in excess oxygen, at 95°.

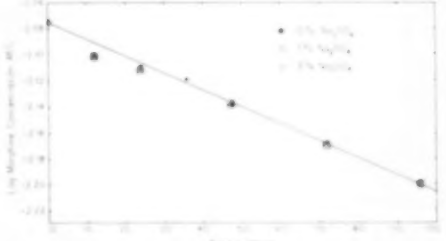


Fig. 7.—The effect of ionic strength on degradation of morphine sulfate in 0.2 *M* acetate buffer, at pH 5.0 and in excess oxygen, at 95°.

ence of atmospheric oxygen. It has been reported (1) that the degradation products of morphine are pseudomorphine and morphine N-oxide together with traces of the base said to be methylamine, and

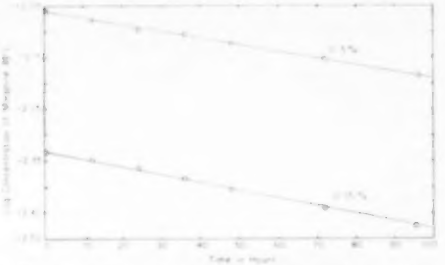


Fig. 8.—Effect of morphine concentration on degradation of morphine sulfate, in excess oxygen, at 95°.

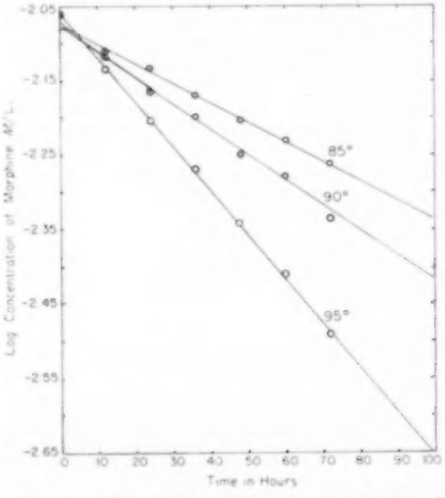


Fig. 9.—Effect of temperature on degradation of morphine sulfate in 0.2 *M* phosphate buffer pH 6.0, in excess oxygen.

that the deterioration of morphine takes place through a condensation process at the phenolic hydroxyl group with the formation of pseudomorphine (10). Derivatives of morphine in which this phenolic group is alkylated, as in codeine, do not undergo this type of oxidation (2). The fact that deterioration of morphine increases rapidly in the presence of ultraviolet light (5) and decreases in a more acidic solution (11) gives additional support to the oxidation reaction. This oxidation of morphine to pseudomorphine may be somewhat analogous to the oxidation of phenols to dimolecular compounds and is presented here in some detail.

The ferricyanide oxidation method commonly used to prepare pseudomorphine involves one electron transfer which is common in free radical systems. This reaction can be illustrated as follows:

$$[Fe(CN)_6]^{3-} + e \rightarrow [Fe(CN)_6]^{4-}$$

Fummerer, *et al.* (20–23), in their studies of the oxidation of naphthols with alkaline potassium ferricyanide reported that the oxidizing agent attacks

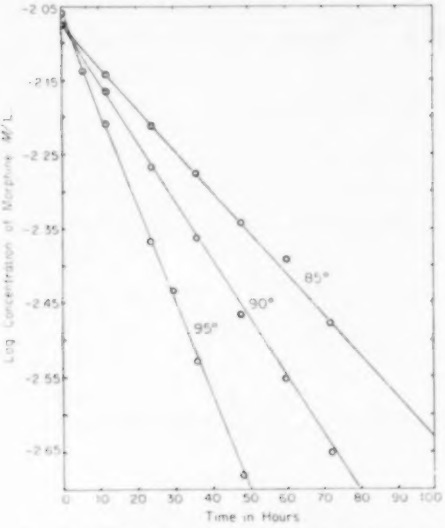


Fig. 10.—Effect of temperature on degradation of morphine sulfate in 0.2 *M* phosphate buffer solution, pH 6.5, in excess oxygen.

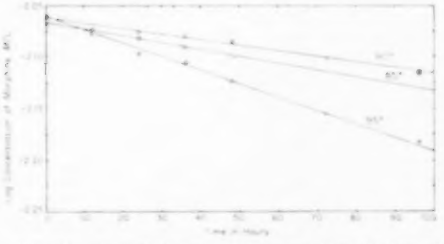


Fig. 11.—Effect of temperature on degradation of morphine sulfate in 0.2 acetate buffer solution at pH 5.0, in excess oxygen.

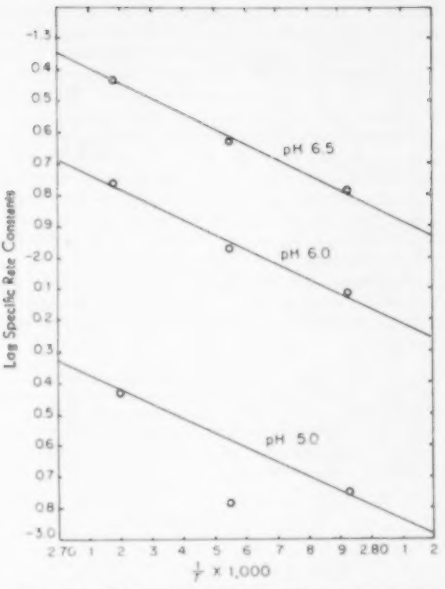


Fig. 12.—Plot of the log specific rate constants as a function of the reciprocal of the absolute temperature.

the OH group and has a direct dehydrogenating action and that the primary oxidation product is a resonant aroxyl radical. They confirmed this by isolating and identifying the possible dimeric forms. The oxidation of naphthol gives binaphthol which is further oxidized to oxy-binaphthylene-oxide and binaphthylene dioxide as illustrated in Diagram I.

Oxidation of *p*-cresol was found to give dicresol and an ether (24, 25). If the resonant forms of *p*-cresol are examined, it becomes evident that these are all cogent in the transition of the resonant aroxyl radical as illustrated in Diagram II.

Considering such a reaction and by direct analogy, morphine could undergo a similar type of process in its degradation and subsequent production of pseudomorphine as shown in Diagram III.

Mechanism of Degradation of Morphine.—Since data obtained from this investigation showed that the rate of decomposition of morphine in solution was dependent on the presence of oxygen and that no decomposition occurred in systems void of oxygen, it was concluded that a free radical reaction was involved in this process. It was also found that the rate of degradation was dependent on the hydrogen ion concentration of the solution since the rate was considerably greater at the higher pH. It is interesting to note here that an "S"-shaped curve, similar to the typical dissociation curve, was obtained when the log of the specific rate constant of these reactions was plotted as a function of pH. This indicated that the degradation was dependent on the type of morphine species present in solution. Based on this information, it appeared that the undissociated morphine molecules undergo oxidation more easily. At the pH values employed in this study and considering the pKa and pKb of morphine, only the protonated and undissociated morphine species are involved. The amount of anionic species of morphine present at these hydrogen ion concentrations is negligible and has been included in the concentration of the undissociated species. Since the rate of decomposition of morphine at lower pH's, i. e., at pH 2.5 and pH 4.0, is nearly the same, this indicated that the protonated morphine species also undergoes oxidation, but at a different rate to that of the free undissociated morphine base.

The undissociated (free base form) and protonated morphine are both oxidized by atmospheric oxygen to give a semiquinone (MO) and a free radical peroxide ($HO_2\cdot$). This semiquinone is further transformed to a free radical quinone (MO·), which can undergo coupling with: (*a*) itself, (*b*) the undissociated morphine, and (*c*) the protonated morphine. Since the amount of activated or free radical morphine species present in the system is small compared to the protonated or free base forms, interaction or union of two such activated species is un-

likely. Interaction of this activated species with the protonated or free base form of morphine is more probable resulting in the formation of the dimer pseudomorphine (PSM) with the simultaneous elimination of a hydrogen-free radical H·. This hydrogen-free radical can then react with the peroxide-free radical ($HO_2\cdot$) to form hydrogen peroxide. The hydrogen peroxide which formed in such a process can react with morphine to form morphine N-oxide (MNO), or may decompose to give water and a free radical oxygen which can also react with morphine base to give the N-oxide. The postulated mechanism is given in Diagram IV.

Derivation of the Rate Equation.—From the postulated mechanism and kinetic data obtained in this investigation the following rate equation may be derived

M_t = M + $^+$HM = total concentration of morphine.
M and $^+$HM represent the undissociated and protonated form of morphine, respectively.
MO and $^+$HMO represent the activated forms of the undissociated and protonated morphine species, respectively.

$$M + O_2 \xrightarrow{k_1} MO\cdot + HO_2\cdot$$

$$^+HM + O_2 \xrightarrow{k_2} {}^+HMO\cdot + HO_2\cdot$$

$$^+HMO\cdot + M \xrightarrow{k_3} PSM + H\cdot$$

$$^+HMO\cdot + {}^+HM \xrightarrow{k_4} PSM + H\cdot$$

$$MO\cdot + M \xrightarrow{k_5} PSM + H\cdot$$

$$MO\cdot + {}^+HM \xrightarrow{k_6} PSM + H\cdot$$

$$H\cdot + HO_2\cdot \xrightarrow{k_7} H_2O_2$$

$$H_2O_2 + M \xrightarrow{k_8} MNO + H_2O$$

Application of the usual steady-state treatment for elimination of the unstable free radical and intermediates gives the rate.

$$-d(M_t)/dt = 3k_1(O_2)(M) + 3k_2(O_2)(^+HM)$$

The dissociation equation of an ampholyte may be represented as follows

$$^+HM \rightarrow M + H^+$$

$$Ka = \frac{(M)(H^+)}{(^+HM)}$$

$$M_t = M + {}^+HM$$

$$M = M_t\left(\frac{Ka}{Ka + H^+}\right)$$

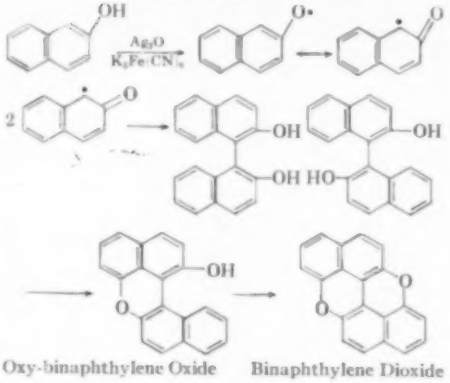
$$^+HM = M_t\left(\frac{H^+}{Ka + H^+}\right)$$

Therefore, the overall rate can be written as

$$-d(M_t)/dt = \left[3k_1(O_2)\left(\frac{Ka}{Ka + H^+}\right) + 3k_2(O_2)\left(\frac{H^+}{Ka + H^+}\right)\right](M_t)$$

DIAGRAM I

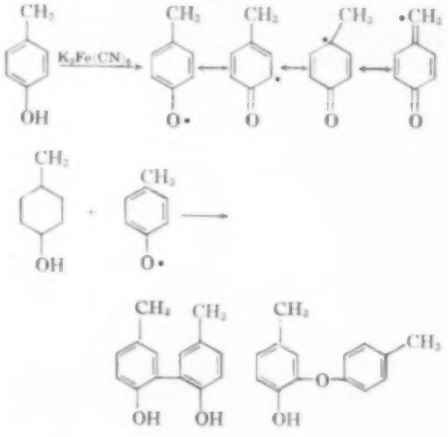
Oxidation of Naphthol (20–23)



Oxy-binaphthylene Oxide Binaphthylene Dioxide

DIAGRAM II

Oxidation of p-Cresol (24, 25)



Since oxygen was maintained in excess, this rate equation becomes pseudo first order.

$$-d(M_t)/dt = \left[k_1'\left(\frac{Ka}{Ka + H^+}\right) + k_2'\left(\frac{H^+}{Ka + H^+}\right)\right](M_t)$$

When a Ka value of 1.7×10^{-7} was chosen for morphine, the values of k_1' and k_2' were calculated and found to be 8.03×10^{-2} per hour, and 1.77×10^{-2} per hour. The success of this derived equation is shown in Table I. It is interesting to note the close agreement of the values obtained using this overall rate equation as compared to the reported experimental data.

CONCLUSION

The degradation of morphine in aqueous solution is dependent on the pH of the solution and on the presence of atmospheric oxygen in the

DIAGRAM III

Formation of Pseudomorphine

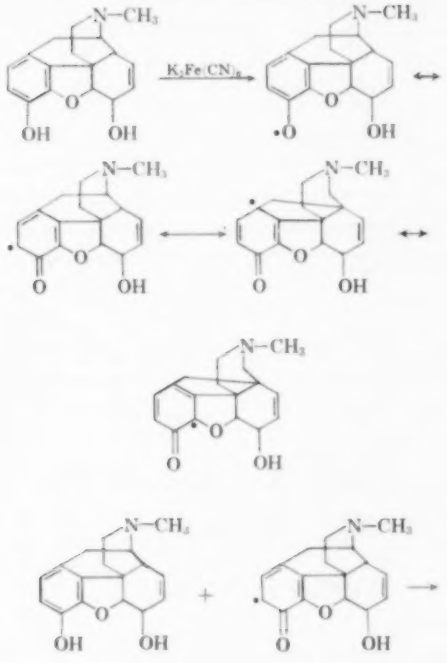


DIAGRAM IV

Degradation of Morphine

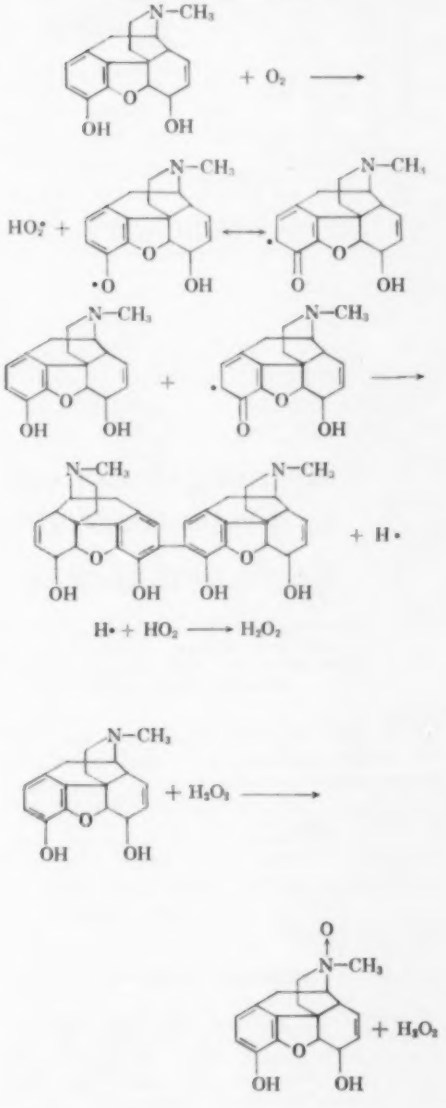


TABLE I.—OBSERVED AND CALCULATED SPECIFIC RATE CONSTANTS AT VARIOUS pH's

pH	$k \times 10^3$ hr.$^{-1}$, Observed	$k \times 10^3$ hr.$^{-1}$, Calcd. by the Derived Eq.	Deviation, %
2.5	1.76	1.77	+0.5
4.0	1.95	1.90	−2.6
4.5	2.16	2.19	+1.4
5.0	2.99	3.08	+3.0
5.5	5.61	5.77	+2.8
6.0	13.78	13.17	−4.6
6.5	29.36	29.23	−0.5
7.0	48.70	51.20	+5.1
		Mean dev.	0.62

system. The overall reaction rate, in systems containing excess oxygen, was found to be equal to

$$\left[k_1'\left(\frac{Ka}{Ka + H^+}\right) + k_2'\left(\frac{H^+}{Ka + H^+}\right)\right](\text{Morphine})$$

The apparent energy of activation, E_a, was calculated to be 22.8 Kcal. for this reaction.

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Antifungal Activity of Some Amides of Dichloroacetaldehyde and Bromal

By WILLIAM D. EASTERLY, Jr., and JAMES E. DUSENBERRY

The fungus-inhibiting properties of a series of amides of dichloroacetaldehyde and bromal were studied. Three of the compounds showed antifungal activity when subjected to tests with *Aspergillus niger* and *Trichoderma viride*. Terminal chlorine atoms appeared to be a factor in enhancing the antifungal activity.

RECENTLY the syntheses of some amides of dichloroacetaldehyde (1) and bromal (2) were reported. In view of the fact that studies have shown the potentialities of certain substituted amides as antifungal agents and have indicated an increase in antifungal activity in certain compounds following halogenation (3, 4), it was decided to subject these compounds to preliminary tests.

EXPERIMENTAL

Procedure.—A study of the fungus-inhibiting properties of these compounds was carried out by a modification of a method used by Bateman (5), Vincent (6), and also by Leonard and Blackford (3). This procedure consists in comparing the growth rates of the test fungus upon nutrient agar containing known concentrations of the compound to be tested with that of a control, identically treated but containing none of the test compound. All compounds were tested in triplicate for each concentration.

Preparation of Culture Medium.—The culture medium, Sabouraud's dextrose agar, was prepared in the usual manner; and prior to being steam sterilized for twenty minutes at 15 pounds pressure, a glass enclosed magnetic stirring bar was added to the flask containing it. When the flask was removed from the autoclave it was placed in a hemispherical heating mantle, which in turn was placed on a magnetic stirring motor. A weighed amount of the test compound was added to the agitated culture medium which was maintained in the liquid state.

Most of the amides of dichloroacetaldehyde, in the indicated concentrations, were readily soluble in the culture medium, while the amides of bromal were difficult to dissolve. The latter could be uniformly dispersed, however, by keeping the magnetic stirring bar moving rapidly, and gradually lowering the temperature of the heating mantle until the consistency of the culture medium was still suitable for pouring, yet keeping the test compound in suspension. The culture medium was then poured into previously chilled, sterile 9-cm. Petri dishes.

Inoculation.—The test organisms to be used had been grown on Sabouraud's agar slants for seven days at 30°. Separate spore suspensions of each of the two test organisms were prepared by washing the agar slants with a 5-ml. portion of normal saline. As an inoculum, 0.03 ml. of this spore suspension was added to the center of the Petri dishes containing test substance, and a like amount was added to the control.

Growth Measurement.—The plates, incubated at 37°, were observed daily and measurements of

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TABLE I.—DICHLOROACETALDEHYDE AMIDES

$RCONHCH(OH)CHCl_2$

Compound[a]	Formula	Inhibition, % Upon *Aspergillus niger*		Inhibition, % Upon *Trichoderma viride*	
		10^{-2} mole/L.	2×10^{-3} mole/L.	10^{-2} mole/L.	2×10^{-3} mole/L.
Acetamide	$C_4H_5Cl_2NO_2$	−4[b]	9	0	10
Phenylacetamide	$C_{10}H_{11}Cl_2NO_2$	22	72	69	100
α-Chloroacetamide	$C_4H_6Cl_3NO_2$	100	100	49	100
Propionamide	$C_5H_9Cl_2NO_2$	17	39	0	0
Isobutyramide	$C_6H_{11}Cl_2NO_2$	0	16	19	26
Isovaleramide	$C_7H_{13}Cl_2NO_2$	14	31	11	23
Benzamide	$C_9H_9Cl_2NO_2$	76	90	69	100

[a] For convenience, the *Chemical Abstracts* nomenclature for these compounds, N-(2,2-dichloro-1-hydroxyethyl) amides, was not used in this table.
[b] This compound, in the 10^{-2} mole/L. concentration, appeared to enhance rather than inhibit the growth of *Aspergillus niger*.

TABLE II.—BROMAL AMIDES

$RCONHCH(OH)CBr_3$

Compound[a]	Formula	Inhibition, % Upon *Aspergillus niger*		Inhibition, % Upon *Trichoderma viride*	
		10^{-2} mole/L.	2×10^{-3} mole/L.	10^{-2} mole/L.	2×10^{-3} mole/L.
Acetamide	$C_4H_6Br_3NO_2$	62	82	23	45
Phenylacetamide	$C_{10}H_{10}Br_3NO_2$	21	24	13	13
α-Chloroacetamide	$C_4H_5Br_3ClNO_2$	100	100	100	100
Propionamide	$C_5H_8Br_3NO_2$	26[b]	23	12	17
Isobutyramide	$C_6H_{10}Br_3NO_2$	35	59	33	38
Phenylbutyramide	$C_{12}H_{14}Br_3NO_2$	13	17	11	11
Benzamide	$C_9H_8Br_3NO_2$	11	42	11	22
Nicotinamide	$C_8H_7Br_3N_2O_2$	59	78	26	43

[a] For convenience, the *Chemical Abstracts* nomenclature for these compounds, N-(2,2,2-tribromo-1-hydroxyethyl) amides, was not used in this table.
[b] The lower concentration appeared slightly more inhibitive than the larger concentration.

growth taken. The values were then plotted against time and the growth rate determined as the slope of the best straight line through the points. Inhibition was then calculated according to the method of LaRocca, Leonard, and Weaver (4) as follows

$$I = \frac{C - T}{C} \times 100\%$$

where I = percentage inhibition; C = growth rate of control, mm./hour; and T = growth rate of toxic, mm./hour.

RESULTS

The inhibition data, calculated from the growth measurements, are presented in Table I and Table II.

Of the dichloroacetaldehyde amides tested, the α-chloroacetamide, the benzamide, and the phenylacetamide derivatives indicated a greater degree of inhibition against both the *Aspergillus niger* and the *Trichoderma viride*. It will be noted that the compound with the highest antifungal activity had the greatest amount of chlorine in its molecule and that this chlorine was on both terminal groups. The other two dichloroacetaldehyde derivatives showing fungus inhibition were aromatic compounds. None of the aliphatic dichloroacetaldehyde compounds showed any appreciable antifungal activity.

Of the bromal amides tested, the α-chloroacetamide derivative gave 100% inhibition in the concentrations used. The acetamide and the nicotinamide derivatives showed greater than 50% inhibition against *Aspergillus niger*, while all of the compounds other than bromal α-chloroacetamide gave less than 50% inhibition against *Trichoderma viride*. Here again the chlorine atom in the amide portion of the molecular structure seemed to be a factor for antifungal activity.

SUMMARY

1. A satisfactory method for determining the fungus-inhibiting properties of some dichloroacetaldehyde and bromal amides is described.

2. The dichloroacetaldehyde derivatives of α-chloroacetamide, benzamide, and phenylacetamide, as well as the bromal derivative of α-chloroacetamide, showed antifungal activity against *Aspergillus niger* and *Trichoderma viride*.

3. Further antifungal screening is being conducted out by The Upjohn Co.

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Rapid, Simple Colorimetric Method for the Determination of Micro Quantities of Gibberellic Acid

By HORACE D. GRAHAM and LINNIE B. THOMAS†

Gibberellic acid reacts with acidic 2,4-dinitrophenylhydrazine to yield a product, presumably the 2,4-dinitrophenylhydrazone of gibberic acid which, when treated with alcoholic potassium hydroxide, gives rise to a stable wine-red color the intensity of which is proportional to the amount of gibberellic acid present when measured at 430 or 540 mμ. The temperature of heating the gibberellic acid with acidic 2,4-dinitrophenylhydrazine is very critical. At 70° very little or no color development occurs. At 80° much longer periods are required for maximum color development on alkali treatment as compared to heating at 100°, where only a five-minute period of heating is necessary for maximum color development. The effect of several variables on the reaction was thoroughly investigated and conditions for reproducibility established. Indoleacetic acid, 2,4-dichlorophenoxyacetic acid and kinetin failed to give the typical wine-red color when heated at 100° for five minutes under identical conditions.

DESPITE A PLETHORA of literature on the effect of gibberellic acid on plants, the mechanism of action of this plant growth regulator has not yet been elucidated. Recently, studies on growth responses resulting from gibberrellic acid and 2,4-dichlorophenoxyacetic acid interaction have been reported (1) and the stability of gibberellic acid to heat at various pH levels has been questioned (2). To facilitate studies on the mechanism of action of gibberellic acid, and for its rapid assay in pure solutions or in the presence of other plant growth regulators, much simpler and more rapid and sensitive tests than those now available (3–7) are desirable. This communication describes an approach to such a need.

Gibberellic acid, when subjected to acid hydrolysis at 100° produces gibberic acid, a ketonic compound. The use of 2,4-dinitrophenylhydrazine to isolate and characterize small quantities of aldehydes and ketones responsible for off-flavors in foods, food products, and other biological materials is well known (8–16). Spectrophotometric procedures based on the alkaline 2,4-dinitrophenylhydrazine method have been applied to the quantitative determination of aliphatic and aromatic carbonyl compounds (17–21) and steroids (22).

Preliminary experiments showed that, with adequate control, the wine-red color produced on the addition of alcoholic potassium hydroxide to the product resulting from heating gibberellic acid with acidic 2,4-dinitrophenylhydrazine at 100° for five minutes could be made the basis of a rapid, simple, reproducible, and highly sensitive test for gibberellic acid, gibberellin, gibberellin A_1, and the potassium salt of gibberellic acid. Moreover, under the prescribed conditions, indoleacetic acid, 2,4-dichlorophenoxyacetic acid, kinetin, and other auxins do not give the characteristic wine-red color.

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† National Science Foundation undergraduate research participant.
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EXPERIMENTAL

Materials.—*Gibberellic acid*, supplied by Eli Lily and Co. and Abbott Chemical Laboratories. *Gibberellin*, supplied by Velsicol Chemical Corp. and Chas. Pfizer and Co., Inc. *Potassium gibberellate*, 75% potassium salt, supplied by Nutritional Biochemicals Corp. *Gibrel*, supplied by Merck and Co. *Gibberellin A_1*, supplied by Abbott Laboratories. Unless otherwise stated, stock 400 mcg./ml. solutions of the above were prepared in carbonyl-free alcohol. When necessary, further dilutions were made with carbonyl-free alcohol. *2,4-Dinitrophenylhydrazine (2,4-DNPH)*, supplied by Eastman Organic Chemicals.

Carbonyl-Free Methyl Alcohol or Carbonyl-Free Ethyl Alcohol.—Five hundred milliliters of the desired alcohol, 5 Gm. of 2,4-dinitrophenylhydrazine, and 1.0 ml. of concentrated hydrochloric acid were refluxed for three hours. The alcohol was distilled, the fraction boiling at 64.7–65° collected and redistilled.

Alcoholic Potassium Hydroxide 10%.—Ten grams of potassium hydroxide (U. S. P. pellets, Fisher Scientific Co.) was dissolved in 20 ml. of double-distilled water and the contents made up to 100 ml. with carbonyl-free alcohol. The 100-ml. volumetric flask used was kept in an ice bath during all additions.

2,4-Dinitrophenylhydrazine Reagent.—Five milli-

liters of concentrated hydrochloric acid was added to 100 mg. of 2,4-dinitrophenylhydrazine and 50 ml. of carbonyl-free alcohol. The mixture was gently heated until all the 2,4-dinitrophenylhydrazine was dissolved, cooled, and diluted to 100 ml. with carbonyl-free alcohol. A new batch of reagent was prepared every five days.

Equipment.—Pyrex, 20-ml., ground-glass-stoppered test tubes, constant temperature water bath or suitable oil bath, volumetric pipets, and Coleman Universal spectrophotometer, model 14.

Procedure.—A 1-ml. portion containing 40–400 mcg. of gibberellic acid or gibberellin was placed in each of seven Pyrex, ground-glass-stoppered test tubes. A control tube containing 1 ml. of carbonyl-free alcohol instead of gibberellic acid was included. One milliliter of the 2,4-dinitrophenylhydrazine reagent was added to each tube and the mixture heated at 100° for exactly five minutes. Zero time was taken as thirty seconds after the immersion of the last tube. At the end of this period, the tubes were rapidly removed and placed in an ice water bath for five minutes. A thick oily droplet condensed and settled to the bottom of the tube. Five milliliters of 10% potassium hydroxide in 80% carbonyl-free methyl alcohol was rapidly added to each tube; the contents were mixed well and allowed to stand for five minutes. A wine-red color developed indicating a positive test. Fifteen milliliters of double-distilled water were added to each tube, the contents mixed well, diluted 1:2 with distilled water, and the intensity of the color measured at 430 and 540 mμ with a Coleman Universal spectrophotometer, model 14, using a reagent blank. The wavelength of maximum absorption was determined by plotting the absorbance as a function of the wave length as shown in Fig. 1. Here the total volume of the colored solution was 20 ml. and distilled water was used as the blank.

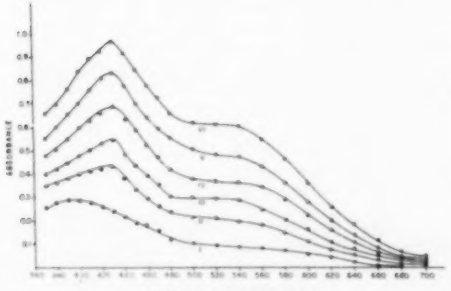

Fig. 1.—Absorption spectrum of color produced by gibberellin in the alkaline 2,4-dinitrophenylhydrazine method. I, 0 mcg./ml.; II, 2 mcg./ml.; III, 4 mcg./ml.; IV, 6 mcg./ml.; V, 8 mcg./ml.; VI 10 mcg./ml.

Over the concentration range employed, Beer's law was obeyed as indicated by the linear relationship resulting when the logarithm of the per cent transmission is plotted as a function of concentration (data in Table I). This relationship can be described by the following logarithmic least squares equations:

$$X_{430} = \frac{2.0031 - \log \% \ T_{\lambda 430}}{0.072124}$$

$$X_{540} = \frac{1.99062 - \log \% \ T_{\lambda 540}}{0.04914}$$

where X = concentration of gibberellin in mcg./ml. and 430 and 540 mμ are the respective wavelengths at which the intensity measurements were made.

Similar equations can be derived for gibberellic acid, gibberellin A_1, and potassium gibberellate.

For the assay of unknown samples, the same procedure can be used after any necessary dilution. The amount of gibberellic acid present can then be determined by use of the standard curve or the logarithmic least squares equation.

TABLE I.—RELATIONSHIP BETWEEN CONCENTRATION OF GIBBERELLIN AND COLOR INTENSITY

Concentration of Gibberellin, mcg./ml.	Transmission, % (Average of Triplicate Readings) At 430 mμ	At 540 mμ
1.0	86.5	88.5
2.0	71.0	78.0
4.0	52.0	61.5
6.0	37.0	50.0
8.0	26.5	40.5
10.0	18.9	31.0

INFLUENCE OF VARIABLES ON COLOR DEVELOPMENT

Development of the wine-red color is dependent on close control of several factors. Since any change of several variables will either influence or totally eliminate color development, it was found necessary to study critically such variables and select appropriate conditions for reproducibility.

Temperature of Heating.—In order to study this variable, 400 mcg. of gibberellin in a volume of 1 ml., and 1 ml. of the 2,4-dinitrophenylhydrazine reagent were treated according to the general procedure at 100, 90, 80, and 70°. The final concentration after dilution with double-distilled water was 10 mcg./ml. (total volume 40 ml.).

The results recorded in Table II indicate that heating for as short a period as five minutes at 100°

TABLE II.—EFFECT OF TEMPERATURE OF HEATING ON COLOR DEVELOPMENT

Duration of Heating, min.	Transmission at 430 mμ, % —Temperature, °C.— 70	80	90	100
1	No color	53	48.0	42.0
3	No color	...	41.5	20.5
5	No color	34.2	29.2	20.0
7	No color	...	28.5	20.2
10	No color	28.5	20.5	20.0
15	No color	26.5	20.0	19.6
20	No color	21.0	19.5	19.8
30	No color	19.5	19.5	20.0
40	No color	19.0	19.0	..
50	No color	19.5	19.8	..
60	Very little color	19.0	19.2	..

Table III.—Influence of Variables upon Color Developed in Test for Gibberellic Acid

Variable	Range Investigated	Limits for Reproducibility Min.	Max.	Value Selected
Concentration of 2,4-DNPH, mg./ml.	0.1–2.0	0.4	2.0	1.0
Concentration of KOH in 80% alcohol, %	1.0–20	2.0	20.0	10.0
Final alcohol concentration, %	1.0–80	...	80	12
Diluent for color developed	H_2O, carbonyl-free MeOH, alcoholic KOH	...	...	H_2O Selected
Acidity of 2,4-DNPH reagent, *N*	0.1–2.0	0.2	1.5	0.5
Color stability	0.0–48 hr.	...	...	10 min. after color development

produced maximum color development. This condition was selected as the most suitable since even after twenty minutes heating at this temperature very little difference in absorbance was noted. Failure of color development at 70° and the comparatively slower color development at 80 and 90° make these temperatures less suitable for rapid work.

Concentration of Potassium Hydroxide.—From Table III, it is evident that under the experimental conditions employed, the concentration of potassium hydroxide employed should be between 5–15%. Below 5%, color intensity was reduced due, apparently, to incomplete alkalinization of the 2,4-dinitrophenylhydrazone. At a concentration of 20%, color intensity increased slightly. In view of the constancy of color intensity when the potassium hydroxide concentration is 5–15%, a concentration of 10% was selected.

Concentration of 2,4-Dinitrophenylhydrazine.—With a maximum final concentration of 10 mcg./ml. of gibberellic acid, in a total volume of 40 ml., 0.4–1.0 mg. of 2,4-dinitrophenylhydrazine should be present in the 1 ml. of the reagent used; 1 mg. per ml. was selected for use. Table III summarizes the results.

Influence of Alcohol Concentration.—When the potassium hydroxide concentration was kept constant at 10%, maximum color intensity was realized at alcohol concentration of 20–80%. At alcohol concentrations below 20% color intensity decreased, and above 80% precipitation of salt occurred. The results are summarized in Table III.

Influence of Acid Strength of the 2,4-Dinitrophenylhydrazine Reagent.—The acidity of the 2,4-dinitrophenylhydrazine reagent proved critical for color development, as shown by Table III. For good color development, the reagent should be 0.2–1.5 *N* with respect to hydrochloric acid. Below 0.2 *N* poor color development, or no color development at all, occurred. Above 1.5 *N* color intensity diminished, as compared to that obtained with 0.2–1.0 *N*. For future experiments a reagent which was 0.5 *N* with respect to hydrochloric acid was chosen.

Stability of the Color Developed.—Under the experimental conditions employed, the color developed was found to be stable for as long as forty-eight hours when allowed to stand at room temperature (28 ± 1°) in ground-glass-stoppered test tubes. Therefore, measurement of color intensity can be made at any time within this period without incurring serious errors.

Selection of a Suitable Diluent for the Color Developed.—For diluents the alcoholic potassium hydroxide, carbonyl-free methanol, or distilled water could be used. With alcoholic potassium hydroxide, further addition may lead to nonreproducibility of results due to salt formation. Carbonyl-free alcohol may also cause difficulties in salt formation, hence water was selected as the most readily available and suitable diluent.

Influence of Water in the Reaction Vessel.—When water was present in the medium, color development was poor or totally eliminated. For this reason, all reagents were diluted with absolute methanol or ethanol. For practical purposes, however, experimental gibberellic acid systems are usually prepared in buffers or systems of low alcohol concentration. By evaporating solutions containing gibberellic acid or gibberellin at 105° for as long as twenty-four hours, then adding 1 ml. of carbonyl-free alcohol to the dry system, the color developed according to the general procedure showed no important deviation from that given in the absolute systems (Table IV). Therefore, this procedure is recommended for direct assay of aqueous systems.

Table IV.—Effect of Dehydration at 105°C. on Color Intensity of Potassium Gibberellate Solutions

Time of Heating, hr.	Transmission, % At 430 mμ	At 540 mμ
1	36.5	55.5
2	20.5	38.5
3	20.6	38.0
4	20.4	38.2
8	20.2	39.0
12.0	20.5	39.0
16.0	20.2	40.0
24.0	22.5	40.5

Influence of Various Salts Commonly Used as Buffers.—Gibberellic acid, when applied to plant systems as sprays or droplets, is usually incorporated in buffers of various sorts. It was, therefore, advisable to assess the effect of these on the intensity of the color developed. One milliliter of each of the buffers listed in Table V was placed into glass-stoppered test tubes. One milliliter containing 400 mcg. of potassium gibberellate dissolved in water was added to each test tube and the contents of the tube evaporated to dryness in an electric oven at 105°. After evaporation, 1 ml. of carbonyl-free methanol and 1 ml. of the 2,4-dinitrophenylhydrazine reagent were added to each test tube and the color developed according to the general procedure.

From Table V it is seen that the citrate and

Table V.—Effect of Various Salts on Color Intensity in the Determination of Potassium Gibberellate by the Alkaline 2,4-Dinitrophenylhydrazine Method

Salt Used	Transmission at 430 mμ, % Normality 0.2	0.10	0.05	0.01	Nature of Color Produced
Ammonium citrate	12.2	14.2	14.6	14.4	Off-color
Ammonium sulfate	20.8	20.2	20.4	20.0	Wine-red
Ammonium acetate	20.0	20.6	20.2	20.6	Wine-red
Disodium hydrogen phosphate	22.0	20.8	20.6	20.2	Wine-red
Sodium chloride	20.4	20.6	20.2	20.0	Wine-red
Potassium acid phthalate	10.0	10.6	11.0	14.0	Off-color
Potassium chloride	22.0	20.6	20.2	20.0	Wine-red
Sodium carbonate	22.0	22.0	21.8	22.0	Slight off-color
Sodium bicarbonate	21.0	21.2	20.4	20.2	Wine-red
Boric acid	20.8	20.6	20.2	20.2	Wine-red
H_2O + potassium gibberellate	20.8	20.2	20.4	20.2	Wine-red
Carbonyl-free MeOH + potassium gibberellate	...	20.4	...	...	Wine-red
Carbonyl-free MeOH only	...	80.4	...	...	...
H_2O only	...	79.8	...	...	...

phthalate ions will interfere rather seriously in color development. Such interference is due to colors produced by the ions themselves on heating and not to any interaction with the gibberellic acid. The absence of interference from several other salts, however, makes it possible to conduct direct assays in a variety of buffer solutions. Tween 20, commonly incorporated into gibberellic acid buffered media, when subjected to the same experimental conditions, gave an intense color which will interfere severely.

Significance of a Small Volume.—Although 5–10 ml. of alcohol can be used with the same ultimate relative results, due mainly to the low-boiling point of methanol, the total volume should preferably be minimized to 0.5–2.0 ml. in order to make the blank reading as low as possible. This objective is also further attained by diluting up to 40 ml. after addition of alkali. In addition, such extensive dilution with water facilitates solution of any salt formed in the alcoholic medium. In cases where the assay of lower concentrations of gibberellic acid must be performed, the procedure can be conveniently scaled down to accommodate determinations.

Relationship Between Concentration of Gibberellin and the Intensity of the Color Developed.—After the conditions for optimal color development were established, aliquots necessary to give final concentrations of gibberellin shown in Table I were placed in duplicate glass-stoppered test tubes and the color developed and measured according to the general procedure.

It can be seen that when the logarithm of the per cent transmission is plotted as a function of the concentration, a straight line results indicating compliance with Beer's law over the concentration range used and that a quantitative relationship exists.

DISCUSSION AND SUMMARY

Mechanism of the Reaction.—Gibberellic acid readily loses carbon dioxide in the presence of hot hydrochloric acid, producing allogibberic acid, from which gibberic acid, a ketonic compound, can result through the Wagner-Meerwin rearrangement (23, 24). Direct production of gibberic acid from gibberellic acid under the influence of hot dilute hydrochloric acid has also been recorded (23). It is, therefore, postulated that the 2,4-dinitrophenylhydrazone formed is that of gibberic acid. This is substantiated by the fact that at 70°, the wine-red color was not obtained and at 80 and 90° much longer heating times were required as compared to heating at 100°. When gibberellic acid was treated with nonacidic 2,4-dinitrophenylhydrazine and alcoholic potassium hydroxide added as in the general procedure, the wine-red color did not develop. Control tubes containing 2,4-DNPH only gave a yellowish-green color on the addition of potassium hydroxide. This constitutes evidence in favor of the proposed mechanism. The characteristics common to the chromogen from gibberellic acid, gibberellin, and the potassium salt of gibberellic acid lend further credence to the postulate that the 2,4-dinitrophenylhydrazone of a common ketonic compound, gibberic acid, is formed. However, final and unequivocal proof of this postulate will depend upon more comprehensive characterization of the hydrazone.

The exact mechanism for formation of the wine-red color of the 2,4-dinitrophenylhydrazones in strong alkali is not known. Friedemann and Haugen (11) think that it is due to substitution of the nitro group in the *para* position of the benzene ring. Chataway and Clemo (25) presented an alternate explanation which has been widely endorsed by other workers (19,26). According to this school of thought, the color change results from the extraction of a proton and the formation of a resonating quinoidal ion.

The data presented indicate clearly that, under the specified conditions, the proposed reaction can be used for a rapid, simple, reproducible, and highly sensitive method for the quantitative determination of gibberellic acid. Since gibberellin, gibberellin A_1, and the potassium salts of gibberellic acid gave positive tests, the method may be used to estimate all these forms. Although the reaction must be carried out in an anhydrous medium, the simple and expedient process of dehydrating aqueous solutions at 105°, without significant destruction of the growth regulator, permits ready assay in experimental buffer systems. In addition, most of the common salts used as buffers do not interfere severely when present

TABLE VI.—SUMMARY OF REACTION OF VARIOUS PLANT GROWTH REGULATORS IN ALKALINE 2,4-DINITROPHENYLHYDRAZINE METHOD

Compound Tested	Production of Wine-Red Color	λ Maximum
Gibberellic acid	+	430, 540
Gibberellin A_1	+	430, 540
Gibrel (dissolved in alcohol)	+	430, 540
Gibrel (dissolved in H_2O)	+	430, 540
Potassium gibberellate (dissolved in H_2O)	+	430, 540
Gibberellin	+	430, 540
Indoleacetic acid	−	390
Indolebutyric acid	−	390
Indolepropionic acid	−	390
Kinetin	−	390
2,4-Dichlorophenoxyacetic acid	−	390
Tryptophan	−	390

in the medium at the tested concentrations of 0.01–0.2*N*. Citrate and phthalate should be avoided as these give rise to severe off-colors. Tween 20, a nonionic surfactant, widely used as an emulsifier in gibberellic acid experimental solutions interferes drastically. All polyoxyethylene surface-active agents should be avoided. If necessary, the Spans, sorbitan-fatty acid surfactants, are recommended.

Negative results obtained with important plant auxins (Table VI) are of particular significance in view of the fact that fundamental studies on gibberellic acid–auxin interactions are now gaining accelerated prominence.

As seen from Fig. 1, color intensity measurements may be made at 430 or 540 mμ. The former peak is the sharper of the two but is relatively closer to the absorption maximum of the blank which is at 390 mμ. Despite its lack of incisiveness, the 540 mμ peak permits measurement in a range where the blank contribution is almost negligible.

The proposed method is suitable for the assay of extracted samples of gibberellic acid or gibberellins and will lend itself to fundamental studies on plant auxin interaction studies.

CONCLUSIONS

The alkaline 2,4-dinitrophenylhydrazine method which has been used for the estimation of carbonyl compounds and steroids, has been successfully adapted to the quantitative estimation of gibberellic acid, gibberellin, gibberellin A_1, and potassium gibberellate. The following, conditions were found to give optimal and reproducible results: heating an anhydrous, alcoholic solution of the growth regulator with 1 ml. of alcoholic 2,4-dinitrophenylhydrazine reagent for five minutes, adding 5 ml. of 10 per cent alcoholic potassium hydroxide, dilution with distilled water, and measuring the color intensity at 430 or 540 mμ against a reagent blank. Direct assay of aqueous or buffer systems can be accomplished after dehydration at 105° for two hours. Most common salts used as buffers do not interfere.

Indoleacetic acid, 2,4-dichlorophenoxyacetic acid, and other plant auxins do not give the characteristic wine-red color. Tween 20 and other polyoxyethylene-type compounds will interfere. The Spans do not interfere.

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Polarography of Iodochlorhydroxyquin and Diiodohydroxyquin

By LEO WAYNE BROWN and EDWARD KRUPSKI

Two iodo-organic compounds, iodochlorhydroxyquin and diiodohydroxyquin, were reduced at the dropping mercury electrode of a polarograph in nonaqueous systems. The diffusion current at various concentrations was determined. The currents produced were found to be diffusion controlled and applicable to a quantitative assay in the range of concentrations used.

THE POLAROGRAPHIC behavior of iodo compounds has been studied by Gergely and Iredale (1). They investigated reduction potentials of the iodobenzoic acids, iodoacetic acid, iodoanilines, and iodophenols in various media and buffers of controlled pH. The reduction potentials of the ionizable compounds that were studied were dependent on pH and the potentials of the unionizable compounds were independent of pH. They found that reduction involved two electrons in most cases. A compound such as *o*-iodobenzoic acid produced a double wave in one medium but only a single wave in another medium. They postulated that the double wave was due to partial dissociation of the compound in that medium. The double wave was diffusion controlled since the sum of the two waves was proportional to the concentration.

Colichman and Liu (2) investigated the polarographic properties of twenty-nine different iodo compounds. Their results were considered in terms of Hammett's linear free-energy relationship between structure and reactivity. They found that the proximity phenomenon of an *ortho* substituent seemed to facilitate the polarographic reduction of the iodo group whether the *ortho* substituent was electropositive or electronegative. They reported half-wave potentials for all the compounds studied.

The relation between structure of aromatic halogen compounds and their reduction potentials was studied polarographically by Levin and Fodiman (3). Their work confirmed what was previously learned from chemical experiments concerning the stabilities of carbon-halogen bonds. They found that a molecule containing chlorine atoms was reduced at a potential independent of whether this molecule was introduced as such or was formed in the solution by reduction of a molecule richer in chlorine (4).

Borrows, Hems, and Page (5) conducted a polarographic determination of thyroxine and 23 related compounds. Page (6) further reported on thyroxine and the radio opaque compounds: iodopyracet, sodium iodomethamate, and iodoalphionic acid.

Neiman, Ryabov, and Sheyanova (7) have reported the half-wave potentials of many halo-organic compounds. They also list the solvent used in each case.

The research already carried out on iodo-organic compounds indicated that polarography might be applied to the assay of two iodo-organic compounds official in the U. S. P., iodochlorhydroxyquin and diiodohydroxyquin. A study of the waves produced by the two compounds would also contribute to the chemical nature of the compounds and further the information as to the ease of removal of iodine from certain organic molecules. From the diffusion current constants obtained, one could correlate similar compounds as to the number of electrons involved in the reduction.

EXPERIMENTAL

Apparatus.—The instrument used was a Sargent model III polarograph equipped with a dropping mercury electrode. The electrolysis cell was of the H type and consisted of a saturated calomel half cell connected to the test solution through an agar bridge. The electrolysis cell was placed in a constant temperature bath maintained at 25 ± 0.2°.

Reagents.—Iodochlorhydroxyquin (Ciba Pharmaceutical Products, Inc.), recrystallized from 95% ethanol to a constant melting point; diiodohydroxyquin (G. D. Searle & Co.), recrystallized from xylene to a constant melting point; lithium chloride (Fisher certified reagent grade); tetraethylammonium bromide (Eastman white label), recrystallized from 90% ethanol; 2-ethoxyethanol (cellosolve, commercial grade) was refluxed with exsiccated ferrous sulfate for thirty minutes, then fractionally distilled and the 133–136° fraction collected.

Solvent-Electrolyte Solution.—The solvent for iodochlorhydroxyquin was buffered ethanol. The buffer was composed of 0.024 *M* potassium acetate and 0.013 *M* acetic acid in 90% ethanol and contained 0.06 *M* lithium chloride as the electrolyte. The pH was determined with a pH meter and adjusted with potassium hydroxide to pH 7.0.

The solvent for diiodohydroxyquin was 2-ethoxy-

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ethanol, containing 0.05 *M* tetraethylammonium bromide as the electrolyte.

Procedure.—Five known solutions of iodochlorhydroxyquin and a blank were prepared with the buffered ethanol solvent. The concentrations were 0.8446, 1.264, 1.656, and 2.507 m*M*. Each solution was placed in the electrolysis cell and deoxygenated by bubbling nitrogen through the solution for thirty minutes. The purified nitrogen was first passed through a solution of the solvent to saturate the gas and decrease the volatilization of the solvent in the electrolysis cell. Each solution was polarographed between zero and minus two volts. The curves of two concentrations, that are typical of all five solutions, are shown in Fig. 1.

Four known solutions of diiodohydroxyquin were prepared with the 2-ethoxyethanol solvent. The concentrations were 1.088, 1.260, 1.510, and 1.762 m*M*. Each solution and a blank were deoxygenated by passing nitrogen through the test solution for thirty minutes, using the same method as given above. Each solution was polarographed between zero and minus two volts. The curves of two concentrations, that are typical of all four solutions, are shown in Fig. 2.

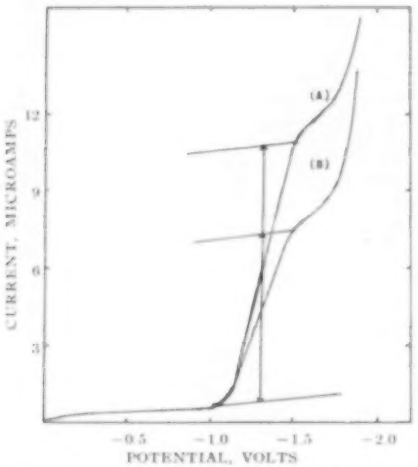

Fig. 1.—Plot of potential *vs.* current of (*A*) 2.507 and (*B*) 1.656 m*M* iodochlorhydroxyquin solution.

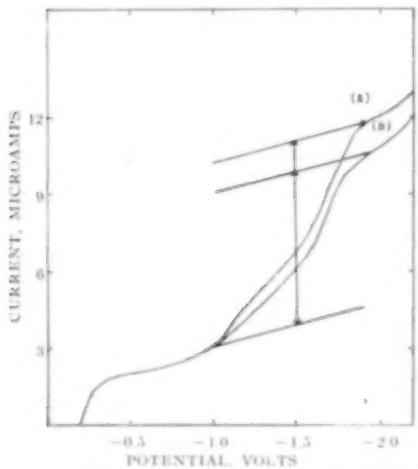

Fig. 2.—Plot of potential *vs.* current of (*A*) 1.762 and (*B*) 1.510 m*M* diiodohydroxyquin solutions.

DISCUSSION

The method of measuring the diffusion currents from the curves obtained, as shown in Figs. 1 and 2, was an arbitrary procedure. All the curves were treated exactly the same way. Two points were selected at the potential where the diffusion current apparently started and ended. From these two points parallel lines were drawn and the vertical distance between them was taken as the diffusion current height. A graph of the diffusion current plotted against concentration was then prepared, and a straight line was drawn through the points. If the line did not pass through the origin the parallel lines of the diffusion current graphs were drawn through the two points at a different angle and the diffusion current height remeasured. This was repeated until the straight line passed through the origin. A graph of diffusion current plotted against concentration is shown in Fig. 3. The experimental error by this method of assaying was found to be ±2%. It was felt that the greatest source of error in the assays was measuring the diffusion current height from the graph of the curves.

The diffusion current for (*B*) in Fig. 1 was 6.56 microamps. The diffusion current constant $(id)/c$ was 6.56/1.656 = 3.96. Practically the same diffusion current constant was obtained for all the other concentrations of iodochlorhydroxyquin. The diffusion current constants for the different diiodohydroxyquin solutions were nearly the same and also the same as the iodochlorhydroxyquin solutions. The diffusion current constants are shown in Table I.

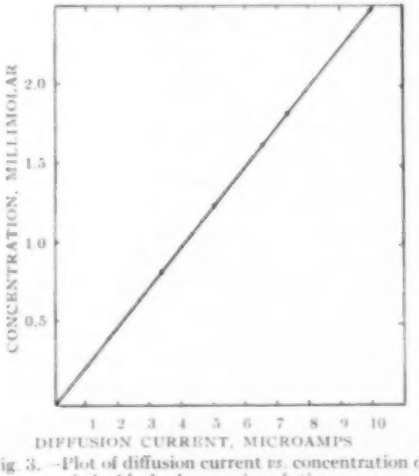

Fig. 3.—Plot of diffusion current *vs.* concentration of iodochlorhydroxyquin solutions.

TABLE I.—THE DIFFUSION CURRENT CONSTANTS FOR DIIODOHYDROXYQUIN AND IODOCHLORHYDROXYQUIN

Concentration, m*M*	*Id*, Microamps	(*id*/*c*)
	Iodochlorhydroxyquin	
0.8446	3.38	4.00
1.264	5.00	3.96
1.656	6.56	3.96
1.899	7.54	3.97
2.507	10.09	4.01
	Diiodohydroxyquin	
1.008	4.03	4.03
1.260	4.87	3.87
1.510	5.85	3.87
1.762	7.15	4.05

SUMMARY

Samples of known concentration of two iodo-organic compounds were reduced at the dropping mercury electrode of a polarograph to determine whether the current thus produced was diffusion controlled and could be used for a quantitative polarographic assay.

A 90 per cent ethanol solution with lithium chloride as supporting electrolyte and potassium acetate-acetic acid buffer was found suitable as the solvent for the polarographic analysis of iodochlorhydroxyquin.

Cellosolve with tetraethylammonium bromide as supporting electrolyte was found suitable for diiodohydroxyquin.

This method of assaying the two compounds was found to be practical in the range of concentrations used. The error of the determination was found to be ±2 per cent.

Similar experimental conditions were maintained through each assay to keep the variables at a minimum. The temperature was held to within ±0.2° by a constant temperature bath but it was felt that the assay could be run at room temperature without a constant temperature bath and still be within the range of experimental error.

The same iodochlorhydroxyquin solution was run on five successive days and no changes in the solution could be detected in that length of time. It was concluded that if the iodo-organic compound solutions were assayed within one or two days after being prepared there would be no appreciable change in results.

Although the iodochlorhydroxyquin produced a single wave while the diiodohydroxyquin produced a double wave, it was concluded that the difference in the solvent was the cause and not that the electrode reaction was different. Gergely and Iredale (1) have shown that changing the solvent can change the wave character of a compound. Other evidence pointing to this conclusion was the fact that the diffusion current constants obtained from both compounds were nearly the same.

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Veratrum Alkaloids XLV

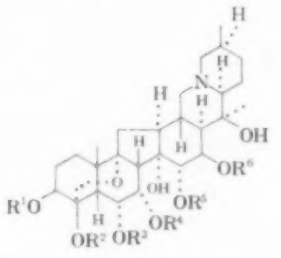
Structure-Activity Relationships in a Series of Protoverine Derivatives

By S. MORRIS KUPCHAN, LAWRENCE C. WEAVER†, C. IAN AYRES, and RUPRECHT H. HENSLER

A study aimed at further elucidation of the relationship between hypotensive activity and structure in a series of derivatives of protoverine is reported. Preliminary pharmacological evaluation of the compounds yielded results which supplement and extend generalizations reported earlier.

TWO ESTER alkaloids derived from protoverine, protoveratrine A and protoveratrine B, have been used in recent years for the treatment of hypertension (1, 2). Recent studies in our laboratory have culminated with the elucidation of the structures and configurations of protoverine (I) (3), protoveratrine A (II) (4), protoveratrine B (III) (4), and several related hypotensive ester alkaloids (5, 6). With the structures of the known natural drugs in hand, systematic elucidation of structure-activity relationships in the series became feasible. Our last report detailed the results of an investigation of the relationship between hypotensive activity and structural isomerism in a series of hypotensive esters of protoverine (7). We report herein studies on a variety of additional derivatives of protoverine which supplement and extend the earlier generalizations.

The preparation of most of the compounds studied in the present investigation has already been reported in earlier papers in the series, and the references to the synthesis of the known compounds are given in Figs. 1–3.[1] Two compounds have not been described previously. Protoverine 3,16-diacetate 6-tosylate 14,15-diisobutyrate (XVI) was prepared by acid hydrolysis of protoverine 14,15-acetonide 3,16-diacetate 6-tosylate (XXXIII) followed by acylation with isobutyric anhydride. Protoverine 14,15-acetonide 6-tiglate (XXXIV) was prepared by acylation of protoverine 14,15-acetonide (8) with tigloyl chloride.



I:	$R^1 = R^2 = R^3 = R^4 = R^5 = R^6 = H$ (3)
II:	R^1 = HMB; $R^2 = R^6 = H$; $R^3 = R^4$ = Ac; R^5 = MB (4)
III:	R^1 = DHMB; $R^2 = R^6 = H$; $R^3 = R^4$ = Ac; R^5 = MB (4)
IV:	R^1 = HMB; $R^2 = R^4 = R^6 = H$; R^3 = Ac; R^5 = MB (6)
V:	R^1 = HMB; $R^2 = H$; $R^3 = R^4 = R^6$ = Ac; R^5 = MB(4)
VI:	R^1 = HMB; $R^2 = H$; $R^3 = R^4$ = Ac; R^5 = MB; R^6 = IB (4)
VII:	R^1 = HMB; $R^2 = R^6$ = IB; R^3 = Ac; $R^4 = H$; R^5 = MB (4)
VIII:	R^1 = HMB; $R^2 = R^4 = H$; R^3 = Ac; R^5 = MB; R^6 = IB (6)
IX:	R^1 = HMB; R^2 = IB; $R^3 = R^4 = R^5 = H$; R^6 = MB (4)
X:	R^1 = HMB; $R^2 = R^3 = R^4 = R^6 = H$; R^5 = MB (4)
XI:	$R^1 = R^2 = R^6 = H$; $R^3 = R^4$ = Ac; R^5 = MB (4)
XII:	$R^1 = R^2 = R^3 = R^4 = R^5$ = Ac; $R^6 = H$ (3)
XIII:	$R^1 = R^3 = R^4 = R^5 = R^6$ = Ac; $R^2 = H$ (3)
XIV:	$R^1 = R^2 = R^3 = R^4 = R^5 = R^6$ = Ac (3)
XV:	$R^1 = R^4 = R^5 = R^6$ = Ac; $R^2 = H$; R^3 = Tos (3)
XVI:	$R^1 = R^6$ = Ac; $R^2 = H$; R^3 = Tos; $R^4 = R^5$ = IB
XVII:	$R^1 = R^2 = R^3 = R^4 = R^5 = H$; R^6 = MB (4)
XVIII:	$R^1 = R^3 = R^5$ = Ac; $R^2 = R^4 = H$; R^6 = MB (4)
XIX:	$R^1 = R^3 = R^4 = R^5$ = Ac; $R^2 = H$; R^6 = MB (4)

Figure 1.

The location of the tiglate at position 6 was assigned on the basis of conversion by periodate (consumption 1.0 mole equivalent) to a product which showed aldehydo-γ-lactone absorption in the infrared, and by analogy to the previously observed facile acylations at C_6 (3, 7).

Thirty-six compounds have been investigated for hypotensive activity and the results are presented in Table I. The methods used have been described (9, 10). Adult mongrel dogs, unselected as to sex, were employed in all experiments described. Anesthesia was maintained at upper plane *ii* or lower plane *i* of Stage III by the ju-

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† Pharmacology Laboratories, Pitman-Moore Co.

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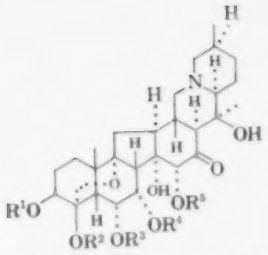
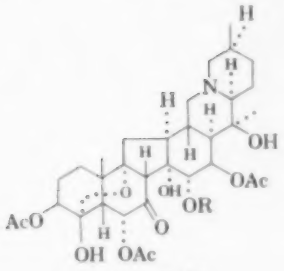
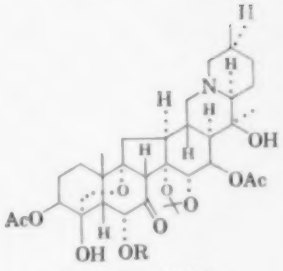
[1] Abbreviations used in Figs. 1–3 and Table I are: Ac = acetyl; IB = isobutyryl; MB = (*d*)-2-methylbutyryl; HMB = (*d*)-2-hydroxy-2-methylbutyryl; DHMB = (*d*)-*threo*-2,3-dihydroxy-2-methylbutyryl; DHMB-Tos = (*d*)-*threo*-2-hydroxy-2-methyl-3-tosyloxybutyryl; Tos = tosyl; Tig = tigloyl; Ade = acetonide; De = dehydro (i. e., keto).



XX: R^1 = HMB; R^2 = H; R^3 = R^4 = Ac; R^5 = MB (4)
XXI: R^1 = DHMB-Tos; R^2 = H; R^3 = R^4 = Ac; R^5 = MB (4)
XXII: R^1 = R^2 = R^3 = R^4 = R^5 = Ac (3)

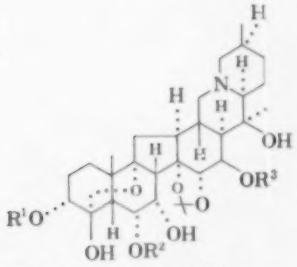


XXIII: R = H (3)
XXIV: R = Ac (6)

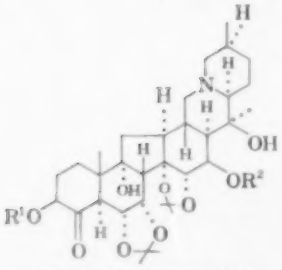


XXV: R = Ac (3)
XXVI: R = Tos (3)

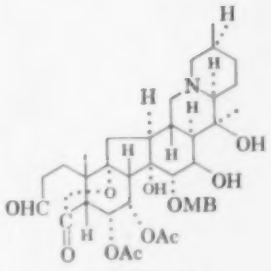
Figure 2.



XXVII: R^1 = R^2 = Ac; R^3 = H (3)
XXVIII: R^1 = R^2 = R^3 = Ac (3)
XXIX: R^1 = R^3 = H; R^2 = IB (3)
XXX: R^1 = R^2 = IB; R^3 = H (3)
XXXI: R^1 = R^3 = Ac; R^2 = IB (7)
XXXII: R^1 = R^3 = H; R^2 = Tos (3)
XXXIII: R^1 = R^3 = Ac; R^2 = Tos (3)
XXXIV: R^1 = R^3 = H; R^2 = Tig



XXXV: R^1 = R^2 = H (3)
XXXVI: R^1 = R^2 = Ac (3)



XXXVII: (4)

Figure 3.

TABLE I.—COMPARATIVE HYPOTENSIVE ACTIVITY OF PROTOVERINE DERIVATIVES IN ANESTHETIZED DOGS

Compd. No.	Substitution at Position							No. Dogs	Dose, γ/Kg.	Blood Pressure: % Chg.	Blood Pressure: Duration, min.	Carotid Occlusion: % Chg.	Carotid Occlusion: Duration, min.
	3	4	6	7	14	15	16						
II	HMB		Ac	Ac		MB		2	2	−49	>83	−68	>90
III	DHMB		Ac	Ac		MB		2	2	−46	26	−55	38
IV	HMB		Ac			MB		3	32	−53	>82	−76	>90
V	HMB		Ac	Ac		MB	Ac	3	128	−14	13	−84	>41
VI	HMB		Ac	Ac		MB	IB	3	128	−36	>60	−54	>50
VII	HMB	IB	Ac	Ac		MB	IB	3	128	−16	6	10	330
VIII	HMB		Ac			MB	IB	2	128	−12	5	8	>30
IX	HMB	IB				MB		2	16	−33	37	−100	<60
X	HMB					MB		4	4	−57	>20	−66	>25
XI			Ac	Ac		MB		2	128	−43	>59	−31	>44
XII	Ac	Ac	Ac	Ac		Ac		6	128	−17	...	...	...
XIII	Ac		Ac	Ac		Ac	Ac	8	128	−15	8	−44	21
XIV	Ac	Ac	Ac	Ac		Ac	Ac	7	128	−13	>19	−21	>21
XV	Ac		Tos	Ac		Ac	Ac	3	128	−24	>35	− 5	>55
XVI	Ac		Tos	IB		IB	Ac	3	128	−16	> 8	−11	>50
XVII						MB		2	128	−12	>45	−17	>45
XVIII	Ac		Ac			MB	Ac	2	128	−11	>10	−12	>54
XIX	Ac		Ac	Ac		MB	Ac	2	128	− 8	8	− 7	>35
XX	HMB		Ac	Ac		MB	De	8	128	− 3	1	...	...
XXI	DHMB-Tos		Ac	Ac		MB	De	3	128	−10	9	−15	45
XXII	Ac	Ac	Ac	Ac		Ac	De	12	128	− 8	3	−21	8
XXIII	Ac		Ac	De			Ac	3	128	−15	<18	− 2	0
XXIV	Ac		Ac	De		Ac	Ac	2	128	−10	10	...	...
XXV	Ac		Ac	De	Ade	Ade	Ac	3	512	− 3	1	...	...
XXVI	Ac		Tos	De	Ade	Ade	Ac	3	128	−12	>15	− 7	60
XXVII	Ac		Ac		Ade	Ade		6	512	− 6	5	...	...
XXVIII	Ac		Ac		Ade	Ade	Ac	5	128	−20	>28	−18	>25
XXIX			IB		Ade	Ade		4	128	− 3	8	...	...
XXX	IB		IB		Ade	Ade		3	128	−15	5	−15	30
XXXI	Ac		IB		Ade	Ade	Ac	3	128	−16	9	−54	53
XXXII			Tos		Ade	Ade		3	128	−12	<20	−18	>60
XXXIII	Ac		Tos		Ade	Ade	Ac	3	128	−16	> 7	−20	>40
XXXIV			Tig		Ade	Ade		3	128	− 6	> 4	− 4	21
XXXV	Isoprotoverine diacetonide							3	128	− 8	5	5	60
XXXVI	Isoprotoverine diacetonide diacetate							3	128	−19	5	−30	>40
XXXVII	Aldehydo-γ-lactone from XI							3	128	− 5	40	−10	15

dicious use of sodium pentobarbital. Test drug solutions were freshly prepared and injected intravenously. The ability of these drugs to lower systemic blood pressure and, in most cases, to decrease the carotid occlusion response have been investigated; the carotid occlusion response is an added indication of activity for this type of compound.

In our previous study (7) the compounds examined were protoverine esters with acetate and/or isobutyrate residues attached at several of the acylable positions (i.e., position numbers 3, 6, 7, 15, and 16). The results supported the following generalizations: (*a*) Esterification at position 16 is accompanied by a profound loss in activity. (*b*) Esterification at positions 3 and 15 is required for high activity. (*c*) Esterification at position 15 with a branched-chain acid is advantageous. (*d*) The ester grouping at position 3 need not be branched. (*e*) Positions 6 and 7 need not be esterified. (*f*) Esterification at position 7 with a branched-chain acid may be disadvantageous.

The following supplementary structure-activity relationships are apparent in the data recorded in Table I:

(*a*) *Oxidation of the alcohol group at position 16 to a ketone group is accompanied by a loss in activity.* Compare XX *vs.* II.

(*b*) *Acetonide formation at positions 14 and 15 is accompanied by a profound loss in activity.* This effect is not explicable in detail at present. The loss in activity may be ascribable to blocking of the 14-hydroxyl group or to absence of an ester function from position 15.

(*c*) *Esterification at position 4 may be disadvantageous.* Compare IX *vs.* X.

(*d*) *Esterification at position 16 with isobutyrate is as disadvantageous as the earlier-noted effect of acetylation at position 16.* Compare VI *vs.* II and VIII *vs.* IV.

(*e*) *The indispensability of esterification at positions 3 and 15 for high activity is further supported by the new data.* Compare XI *vs.* II and XVII *vs.* X.

(*f*) *The generalization that positions 6 and 7 need not be esterified is further supported by the new data.* Compound X was highly active. In

addition, a refinement of the generalization is suggested. While absence of esterifying groups from both positions 6 and 7 is not disadvantageous, absence of an ester from position 7 alone appears to result in a decrease in activity. In other words, 3,6,15-triesters appear to have activity lower than either the corresponding 3,6,7,15-tetraesters or the corresponding 3,15-diesters. Compare IV *vs.* II and X. Support for the added generalization is suggested in a comparison of activity of protoverine 3,15-diisobutyrate 6-acetate with those of protoverine 3,15-diisobutyrate 6,7-diacetate and protoverine 3,15-diisobutyrate (7), and in the apparent low activity of desacetylprotoveratrine B ("desacetylneoprotoveratrine") relative to that of protoveratrine B ("neoprotoveratrine") (6, 11, 12). Further experiments on this point are now in progress and will be reported in due course.

The data presented herein further elucidate the effects of alterations of the protoveratrine molecule on hypotensive activity. A future report will present data obtained in an effort to determine whether any of the more potent compounds possessed a greater margin of safety between the hypotensive and emetic activities than that observed for protoveratrine A.

EXPERIMENTAL

Melting points are corrected for stem exposure. Values of $[\alpha]_D$ have been approximated to the nearest degree. Microanalyses were carried out by Dr. S. M. Nagy and his associates at the Massachusetts Institute of Technology on samples dried under reduced pressure at 110°.

Protoverine 3,16-Diacetate 6-Tosylate 7,15-Diisobutyrate (XVI).—Protoverine 14,15-acetonide 3,16-diacetate 6-tosylate (3) (900 mg.) in dilute hydrochloric acid (0.5 cc. of concentrated hydrochloric acid in 23 cc. of water) was allowed to stand at room temperature overnight. The solution was made alkaline with ammonium hydroxide and extracted exhaustively with chloroform. The chloroform extract was evaporated to dryness under reduced pressure, and the residue was dissolved in pyridine (2 cc.), treated with isobutyric anhydride (3.5 cc.), and heated on the steam bath for four and one-half hours. The solution was cooled, treated with ice water and ammonium hydroxide, and extracted exhaustively with chloroform. The chloroform solution was dried over anhydrous sodium sulfate and evaporated to yield a resin. The resin was chromatographed on Merck acid-washed alumina (15 Gm.) and yielded to ether and to 50% chloroform-ether yellow oily materials. Elution with chloroform yielded a white solid (230 mg.) which was crystallized from acetone-petroleum ether as colorless needles (80 mg.), m. p. 219–221° (decompn.) $[\alpha]_D^{26}$ −60° (c, 1.00, pyr.).

Anal.—Calcd. for $C_{46}H_{65}NO_{15}S$: C, 61.11; H, 7.25; S, 3.55. Found: C, 61.57; H, 7.38; S, 3.40.

In a volatile acid determination (12) 20.44 mg. of the compound yielded an amount of acid equivalent to 12.25 cc. of 0.007317 *N* sodium thiosulfate; calcd. for 2 mole equivalents of acetic acid and 2 mole equivalents of isobutyric acid as required by structure XVI, 12.31 cc.

Protoverine 14,15-Acetonide 6-Tiglate (XXXIV).—A solution of protoverine 14,15-acetonide (8) (3 Gm.) in pyridine (75 cc.) was cooled to 0° and treated with tigloyl chloride (0.7 cc., *ca.* 1.2 mole equivalents). The solution was heated in a water bath at 80° for three hours and was then allowed to stand at room temperature overnight. After basification with dilute ammonium hydroxide, the solution was extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and concentrated to about 15 cc., whereupon 2.05 Gm. of protoverine acetonide separated. The filtrate was evaporated to yield a resin which was chromatographed on Merck acid-washed alumina (25 Gm.). The column yielded to chloroform a solid which was crystallized from acetone-petroleum ether as rectangular prisms (160 mg.), m. p. 180–190°; $[\alpha]_D^{26}$ + 7° (c, 1.00, pyr.).

Anal.—Calcd. for $C_{38}H_{55}NO_{13} \cdot {}^1/_2H_2O$: C, 64.00; H, 8.28. Found: C, 64.02; H, 7.91.

In a volatile acid determination (13) 23.92 mg. of the compound yielded an amount of acid equivalent to 3.78 cc. of 0.009388 *N* sodium thiosulfate; calcd. for 1 mole equivalent of acetic acid as required by structure XXXIV, 3.88 cc.

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Comparison of the Antibacterial Activity of Erythromycin in Various Suppository Bases

By CHARLES W. BLISSITT†, RANDALL B. TINKER, and WILLIAM J. HUSA

A comparison of the antiseptic effect and stability of erythromycin in several suppository bases has been made. It appears that the base influences the effectiveness and the stability of the incorporated antibiotic.

SUPPOSITORIES have been employed as a dosage form for the administration of medicinal agents since ancient times. At one time, little consideration was given to the effect of the base on the drug. However, in recent years interest has developed in the activity and stability of the medicinal agents in the suppository bases.

EXPERIMENTAL

Materials.—Powdered erythromycin base[1] having a potency of 950 mcg. per mg. was used. Suppository base ingredients were of official grade. Dehydrated Penassay seed agar[2] was used as the medium. The inoculum was prepared from a twenty-four-hour culture of *Micrococcus pyogenes* var. *aureus*, ATCC 10,537, (*Staphylococcus aureus*) standardized, and 1 ml. of the suspension was added to each 100 ml. of seed agar at 45°. In the determinations of antiseptic activity, 20 ml. of seed agar was placed in each Petri dish containing four sterile glass cylinders of 8 mm. diameter; these cylinders were removed after the agar had hardened. Ten milliliters of seed agar was added in each Petri dish in the stability tests. A master batch of 84 suppositories was prepared from each of the following bases with sufficient erythromycin base being added to yield a concentration of 50 mg. of erythromycin base per suppository:[3] cocoa butter (base I); cocoa butter with 1% Aerosol OT (base II); Carbowax 1500 and 4000, equal parts (base III); and polyoxyethylene stearate with 3% white wax, 5% water, and 0.5% Aerosol OT (base IV). Duplicate batches of each base were prepared for the purpose of reruns.

The four bases were tested both for antiseptic activity and stability initially and at intervals of one, three, and five months. Bases I and II were stored in a refrigerator at 0–5°, while bases III and IV were stored both at refrigerator and room temperatures (17–20°).

Antibacterial Effect.—Suppositories were formed in a mold which yielded a suppository 3 cm. long and 8 mm. in diameter except for the bullet-shaped tip. Five 3-mm. slices were obtained from each suppository commencing 1 cm. from the tip and working toward the base. Four 3-mm. slices from each of six suppositories (randomly selected) were used as a sample. Each 3-mm. slice was introduced into the cup in the seeded agar plate by means of chilled sterile forceps, no one plate containing more than one 3-mm. slice from the same suppository. After the incubation period of twenty-two hours at 37° the zones of inhibition were measured by means of a divider since the inhibition zones were too large to use the Lilly-Fisher zone reader. One point of a divider was placed at the edge of the zone of inhibition and the divider was extended across the diameter of the cup to the edge of the zone of inhibition on the opposite side. Readings were then made directly from a graduated scale. In order to determine the width of the zone of inhibition, the

TABLE I.—SIZE OF ZONES OF INHIBITION PRODUCED AT DIFFERENT TIMES OF AGING BY 3-MM. SECTIONS OF ERYTHROMYCIN SUPPOSITORIES STORED UNDER REFRIGERATION AND AT ROOM TEMPERATURE

	Elapsed Time in Months							
	0		1		3		5	
Base No.	Orig., mm.	Rerun, mm.	Orig., mm.	Rerun, mm.	Orig., mm.	Rerun, mm.	Orig., mm.	Rerun, mm.
Refrigerated Storage								
I	6.4	8.7	8.8	9.6	10.9	11.4	11.8	13.0
II	5.9	9.4	8.1	9.6	10.9	11.8	11.9	13.1
III	All plates completely clear of growth							
IV	12.0	8.8	12.7	11.8	12.3	12.0	12.1	12.3
Room Temperature Storage								
III	All plates completely clear of growth							
IV	12.0	8.8	12.6	11.7	12.3	11.5	12.1	12.1

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This paper is based upon a dissertation presented to the Graduate Council of the University of Florida by Charles W. Blissitt, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[1] The powdered erythromycin base used in this work, lot number 701869, was graciously supplied by Eli Lilly and Co., Indianapolis, Ind.

[2] The Penassay seed agar was obtained in dehydrated form from the Difco Laboratories, Inc., Detroit, Mich.; lot number 8263.

[3] It is realized that erythromycin preparations currently appearing on the open market contain either 125 or 250 mg. of antibiotic per dosage unit in order that adequate blood levels may be attained. In this investigation a 50-mg. content was used for convenience in preparing the various test solutions described elsewhere in this paper.

TABLE II.—STATISTICAL EVALUATION OF MEAN CONTENT OF ERYTHROMYCIN SUPPOSITORIES MADE FROM DIFFERENT BASES AFTER AGING PERIODS OF ONE MONTH, THREE MONTHS, AND FIVE MONTHS UNDER REFRIGERATION AND AT ROOM TEMPERATURE[a]

Base Number	Elapsed Time, Mo.	Mean Content of Erythromycin in Assay Solution, mg./ml.	$f_{0.05}$	$f_{Calcd.}$	Significant Difference No	Significant Difference Yes	Mean Content Erythromycin Per Suppos., mg.
			Refrigerated				
I	0	1.53	1.664	..	..	..	47.7
	1	1.57	1.664	1.556	x	..	48.2
	3	1.49	1.664	1.556	x	..	46.8
	5	1.49	1.664	1.556	x	..	46.8
II	0	1.25	1.664	..	..	..	46.3
	1	1.26	1.664	0.866	x	..	47.1
	3	1.25	1.664	..	x	..	46.3
	5	1.24	1.664	0.866	x	..	45.9
III	0	1.72	1.664	..	..	..	49.5
	1	1.74	1.664	0.587	x	..	50.1
	3	1.74	1.664	0.587	x	..	50.1
	5	1.71	1.664	0.294	x	..	49.2
IV	0	1.79	1.664	..	..	x	47.9
	1	1.68	1.664	3.891	..	x	44.9
	3	1.61	1.664	5.748	..	x	43.1
	5	1.54	1.664	7.907	..	x	41.2
			Room Temperature				
III	0	1.72	1.664	..	..	..	49.5
	1	1.74	1.664	0.587	x	..	50.1
	3	1.73	1.664	0.294	x	..	49.7
	5	1.67	1.664	1.469	x	..	48.0
IV	0	1.79	1.664	..	..	..	47.9
	1	1.64	1.664	4.789	..	x	43.9
	3	1.53	1.664	7.907	..	x	40.9
	5	1.37	1.664	14.093	..	x	36.7
			Refrigerated (Duplicate Run)				
I	0	1.58	1.664	..	..	..	48.0
	1	1.58	1.664	..	x	..	48.0
	3	1.57	1.664	0.426	x	..	47.7
	5	1.55	1.664	1.277	x	..	47.1
II	0	1.53	1.664	..	..	..	47.5
	1	1.51	1.664	0.639	x	..	46.9
	3	1.51	1.664	0.639	x	..	46.9
	5	1.49	1.664	0.739	x	..	46.3
III	0	1.72	1.664	..	..	..	48.2
	1	1.73	1.664	0.369	x	..	48.5
	3	1.71	1.664	0.369	x	..	47.9
	5	1.70	1.664	0.739	x	..	47.6
IV	0	1.67	1.664	..	..	..	47.6
	1	1.57	1.664	4.556	..	x	44.8
	3	1.51	1.664	7.291	..	x	43.0
	5	1.46	1.664	9.575	..	x	41.6
			Room Temperature (Duplicate Run)				
III	0	1.72	1.664	..	..	..	48.2
	1	1.70	1.664	0.739	x	..	47.6
	3	1.69	1.664	1.277	x	..	47.4
	5	1.68	1.664	1.556	x	..	47.1
IV	0	1.67	1.664	..	..	..	47.6
	1	1.51	1.664	7.291	..	x	43.0
	3	1.46	1.664	9.575	..	x	41.6
	5	1.28	1.664	17.882	..	x	36.5

[a] Sample size, 96.

diameter of the cup (8 mm.) was subtracted from the reading, and the difference was divided by two. This gave the distance from the edge of the cup to the periphery of the zone of inhibition.

Stability Determination.—Four suppositories were selected at random from each master batch of suppositories representing the four bases and each suppository was dissolved in enough solvent to make 25 ml. After solution was effected, an 0.05-ml. aliquot was transferred to sterile disks[4] by the use of a Kahn pipet. The solvent was allowed to evaporate spontaneously and the disks were placed on the surface of plates containing 10 ml. of seeded agar. These plates were incubated at 37° for twenty-two

[4] Schleicher and Schull analytical filter paper disks No. 740-E, 13 mm. in diameter, were used.

hours, after which time the zones of inhibition were read by means of a Lilly-Fisher zone reader.

Standard Curve.—Concurrently with the stability determination experiments, paper disks were prepared from the following concentrations of erythromycin base in acetone: 0.5 mg./ml., 1.0 mg./ml., 2.0 mg./ml., and 4.0 mg./ml. An 0.05-ml. aliquot of each solution was placed on separate paper disks and the solvent permitted to evaporate spontaneously. Four disks, one of each concentration, were placed on the surface of each of 24 plates containing 10 ml. of seeded agar as previously described.

Evaluation of Results.—In the determination of antibacterial activity, the agar cup-plate method of the United States Food and Drug Administration (1) was employed. If a zone of inhibition was produced, the suppositories were considered to have antibacterial activity.

The zones of inhibition obtained from the standard curve plates were plotted against the logarithm of the concentration of erythromycin. A straight line relationship resulted and the zone sizes obtained from the erythromycin suppository disks in the stability tests were converted to mg. by referring to the standard curve. The results obtained were subjected to a statistical analysis employing the Student-Fisher "*t*" at a 95% confidence level to determine the significance of any difference between two mean values.

Selection of Erythromycin Concentration.—The following concentrations in mg. of erythromycin per ml. of acetone were prepared: 0.5, 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0. An 0.05-ml. aliquot was transferred to 12 individual disks and the solvent allowed to evaporate spontaneously. The disks were then placed on seeded agar plates, four disks per plate, each of a different concentration. The disks were run against the test organism as previously described. The zones of inhibition were recorded and averaged; the results were plotted on semilogarithmic paper. The resulting curve was examined to determine the concentration at which a minimal change in erythromycin concentration produced a maximal change in the size of the zone of inhibition. This concentration was determined to be between 0.5 and 1.0 mg. Because of the possibility of decomposition or inactivation of the antibiotic material by heat during the fusion process of preparation, it was decided to employ a concentration of erythromycin which would give a working concentration of 2 mg./ml. when an entire suppository was dissolved in sufficient acetone to make 25.0 ml.

DISCUSSION AND DATA

A study has been made of several representative suppository bases to determine the suitability of the several bases for erythromycin.

The results in Table I show that all four bases produced good zones of inhibition during a five-month period of testing. It can be seen that bases I and II (essentially cocoa butter) gave smaller zones than those produced by bases III and IV which were wholly or partially water miscible.

Summaries of the results obtained from the stability tests on both the original and duplicate experimental batches of erythromycin suppositories are shown in Table II. Examination of the data in Table II shows that there was no significant change in the antibiotic content of suppositories prepared from bases I, II, and III over a five-month period. Suppositories prepared from base IV, however, showed a significant loss of antibacterial activity after one month. This was expected since base IV contained white wax which has been shown to affect erythromycin activity adversely (2). The loss of activity was not as great when erythromycin suppositories in base IV were stored under refrigeration as it was at room temperature.

SUMMARY

1. Four suppository bases, cocoa butter (base I), cocoa butter with one per cent Aerosol OT (base II), Carbowax 1500 and 4000, equal parts (base III), and polyoxyethylene stearate with 3 per cent white wax, 5 per cent water, and 0.5 per cent Aerosol OT (base IV) have been evaluated as vehicles for erythromycin.

2. All bases exhibited an antiseptic activity when tested over a five-month period.

3. Erythromycin suppositories made from bases I, II, and III showed no significant loss of antibacterial activity during a five-month aging period. Base IV, however, showed a significant loss after one month of storage.

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Some Apparent Drug-Vitamin Interrelationships in *Lactobacillus leichmannii* and *Tetrahymena pyriformis*

By J. M. McLAUGHLAN, K. G. SHENOY†, and J. A. CAMPBELL

Forty-four drugs were screened for possible antivitamin effects using *Lactobacillus leichmannii* and *Tetrahymena pyriformis* as test organisms. Tests were done with several concentrations of both drugs and vitamins. The effect of a drug on the response of the test organism to individual vitamins served as the criterion of antivitamin activity. Phenformin appeared to have antifolic acid activity for *L. leichmannii* but not for the protozoan. Chlorpromazine, promazine, chloroquine phosphate, methapyrilene hydrochloride, and methantheline bromide appeared to have antipyridoxal activity for *T. pyriformis*. Propylthiouracil, tolbutamide, metahexamide, and chlorpropamide were found to have antipantothenate activity for the protozoan but not for *L. leichmannii*. The present data suggest that the hypoglycemic action of the sulfonylurea drugs may be related to interference with pantothenic acid metabolism.

IN THE TREATMENT of patients with new chemotherapeutic agents the possibility of undesirable side effects, such as antivitamin activity, must be kept in mind. Isoniazid, for instance, produces an apparent pyridoxine deficiency in a wide variety of biological systems including man and therapeutic amounts of pyridoxine have been recommended during treatment with this drug (1). It is reasonably well established also that primidone and possibly other barbiturates (2) produce a megaloblastic anemia in man which is alleviated by treatment with folic acid or occasionally by vitamin B_{12}. In view of the possible existence of other drug-vitamin interrelationships there is obviously a need for screening methods for testing new drugs for antivitamin effects.

Microbiological systems have been used extensively for studying the metabolism of potentially useful drugs, particularly folic acid antagonists and drugs interfering with the metabolism of purines and pyrimidines (3, 4). A recent large scale collaborative assay (5) demonstrated the usefulness of *Tetrahymena pyriformis* and the lactic acid bacteria for investigating potential antitumor agents. In the present study *T. pyriformis* and *Lactobacillus leichmannii* were used to screen 44 drugs which are used in man over extended periods for possible antivitamin effects. A preliminary report of the findings with the sulfonylurea drugs has already been published (6).

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EXPERIMENTAL

Sterilization of Drugs

Drugs which were only slightly soluble in water were dissolved in dilute hydrochloric acid, dilute sodium hydroxide, or ethyl alcohol. The solutions of drugs were sterilized by filtration through sintered glass but, in a few instances, suspensions of drugs were merely "pasteurized" by heating to 70° for a few minutes.

Screening Tests

The general procedure for detecting antivitamin effects was essentially the same for both test organisms. The toxicity of each drug for the test organism was determined in the optimal medium (i. e., all vitamins in excess of that required for maximum growth) by measuring the effect of several concentrations of drug on the amount of growth after a fixed incubation period. The data relating amount of growth and drug concentration were plotted and the maximum concentration of drug producing no inhibition of growth was estimated (this value was only approximate and varied from day to day). The effect, on individual vitamin assays, of the drug at the highest concentration estimated to be noninhibitory in the optimal medium was determined using single dose assays. An appropriate series of control tubes, to which water was added aseptically instead of a drug, was set up for each test. All tubes were placed in a random order during incubation.

Since there was some variation in the amount of growth (as judged by turbidity) obtained from day to day, even with the media containing optimal amounts of all vitamins, growth was expressed as a per cent of the amount of growth in the control series:

$$\frac{\text{Absorbance of test cultures}}{\text{Absorbance of control cultures}} \times 100$$

Tests in which a drug appeared to affect the growth with suboptimal but not with the optimal concentration of a vitamin were repeated at least once.

Tests with L. leichmannii 313 (ATCC 7830).—Cultures of *L. leichmannii* were maintained and inocula prepared as outlined in the U. S. P. vitamin B_{12} assay (7) except that 0.9% sodium chloride was used for washing and suspending the inoculum. The U. S. P. vitamin B_{12} assay medium containing 0.5 mmcg. B_{12}/ml. was designated as the optimal medium. All media were autoclaved for five minutes at 121° and sterile solutions of drugs were added aseptically. Cultures were incubated for sixteen hours at 37° and growth was measured at 620 mμ in a Coleman model 11 spectrophotometer. The basal media for vitamin assays were the same as the optimal medium except for the vitamin under test and the concentrations of test vitamins (suboptimal) were 17, 4, 0.08, and 0.01 mmcg./ml. for the niacin, pantothenic acid, folic acid, and vitamin B_{12} assays, respectively.

Tests with T. pyriformis W.—Cultures of this protozoan were maintained by weekly transfer in a medium containing 2% peptone and 0.1% yeast extract. The medium designated as optimal was that of Dewey, *et al.* (8), and the same medium except for the concentration of the test vitamin was used for individual vitamin assays. In niacin, pantothenic acid, folic acid, thiamine, riboflavin, and pyridoxal assay media the concentrations of these vitamins (suboptimal) were 10, 20, 0.2, 0.5, 2, and 2.4 mmcg./ml., respectively. The media (minus the vitamin under test) were autoclaved for five minutes at 121° and sterile solutions of vitamins and drugs were added aseptically to give a total volume of 4.0 ml. per 20 mm. tube. Tubes of media were inoculated with 0.2 ml. of inoculum prepared from a three-day-old washed culture, which was diluted 1 to 10 in single strength medium (vitamins omitted). Assay cultures were incubated in a sloped position at 25° for seventy-two hours unless otherwise specified. Growth was measured turbidimetrically at 660 mμ in a Coleman model 11 spectrophotometer after adding 2 ml. of water to each tube. Cultures grown in the presence of each drug were examined microscopically after one, twenty-four, forty-eight, and seventy-two hours of incubation for loss of motility or abnormal morphology.

RESULTS AND DISCUSSION

Estimation of Maximum Noninhibitory Concentrations of Drugs.—The toxicity of each drug for *L. leichmannii* and *T. pyriformis* in the optimal media was determined; the toxicity curves for three drugs for *L. leichmannii* are shown in Fig. 1. These curves were used to estimate the maximum noninhibitory concentrations of each drug, which were used subsequently in the drug-vitamin screening tests. The maximum noninhibitory concentrations of chlorpromazine, promazine, and phenobarbital appeared to be approximately 10, 20, and 80 mcg./ml., respectively. The maximum noninhibitory concentrations of all drugs tested with one or both organisms are given in Table I. In general, most drugs were more toxic for *T. pyriformis* than for *L. leichmannii* although growth of the latter was markedly inhibited by the antifolic acid drugs, aminopterin and pyrimethamine.

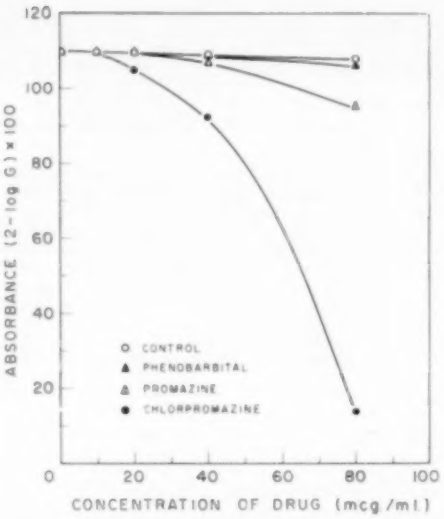

Fig. 1.—The inhibitory effects of three drugs on the growth of *L. leichmannii* in the optional medium.

Screening Tests for Antivitamin Effects.—The inhibitory (or stimulatory) effect of a drug on individual vitamin assays served as the basis of the proposed screening test and the sensitivity of the method was assessed using known antivitamins. The possibility of using one medium (universal medium) equally deficient in several vitamins was also studied; results have been combined with those from vitamin assays and are given in Table II. Pantoyltaurine, a known antagonist of pantothenic acid for lactic acid bacteria, inhibited growth of *L. leichmannii* quite markedly in the pantothenic acid assay but no effect was evident in the "universal medium." *T. pyriformis* was not affected by pantoyltaurine and therefore behaved like higher animals toward this antagonist. The inhibitory effect of 6-aminonicotinamide, a niacin antagonist, was evident in vitamin assays with both test organisms. Pyrimethamine inhibited growth almost completely in each of the three screening systems. Isoniazid was not tested with *L. leichmannii* as this particular culture did not appear to have a requirement for any of the vitamin B_6 factors, but its antivitamin B_6 activity was very evident with *T. pyriformis*. It was apparent from these and other studies (unpublished data) that a screening assay using the universal medium was not sensitive enough for routine use, but antivitamin effects were readily detected in regular vitamin assays.

The drugs listed in Table I were screened for antivitamin effects with *L. leichmannii* and *T. pyriformis* and the results which were thought to be of interest are given in Table III. Some tests indicated possible antivitamin effects. Phenformin and possibly diphenhydramine appeared to be toxic for *L. leichmannii* with the suboptimal concentration of folic acid. Although proguanil hydrochloride (Paludrine) is structurally similar to phenformin and is antago-

TABLE I.—MAXIMUM NONINHIBITORY CONCENTRATION OF DRUGS FOR THE TEST ORGANISMS

Drug	*T. pyriformis* W, mcg./ml.	*L. leichmannii*, mcg./ml.
Acetylsalicylic acid	25	40
6-Aminonicotinamide	100	100
Aminopterin sodium	100	1
Aminopyrine	50	100
Amphetamine	10	80
Aprobarbital	50	50
Barbital	100	150
Butabarbital	50	100
Butallylonal	10	100
Butethal	50	100
Chloroquine phosphate	10	200
Chlorothiazid	10	50
Chlorpheniramine maleate	10	100
Chlorpromazine hydrochloride	1	10
Chlorpropamide	100	80
Cyclopal	25	100
Diphenhydramine hydrochloride	10	100
Glutethimide	25	80
Hexethal	5	..
Hydralazine hydrochloride	5	40
Hydroxyzine	10	50
Isoniazid	25	100
Metahexamide	100	100
Methantheline bromide	200	100
Methapyrilene hydrochloride	25	75
Pantoyltaurine	1000	100
Pentobarbital sodium	10	150
Phenformin	25	100
Phenobarbital sodium	50	200
Primidone	100	100
Probarbital	100	100
Proguanil hydrochloride	10	50
Promazine hydrochloride	1	20
Propallylonal	25	100
Propylthiouracil	50	50
Pyrimethamine	5	1
Quinine sulfate	10	200
Secobarbital sodium	10	50
Sigmodal	25	100
Thialbarbitone	5	..
Thiopental sodium	5	100
Thiopropazate dihydrochloride	10	25
Tolbutamide	50	100
Vinbarbital	100	100

TABLE II.—EFFECT OF ANTIVITAMINS ON GROWTH OF *L. leichmannii* AND *T. pyriformis* WITH LIMITING CONCENTRATIONS OF VITAMINS

Drug	Concn., mcg./ml.	Growth Response[a] *L. leichmannii* Vitamin Assay Media	Growth Response[a] *L. leichmannii* Universal Medium	Growth Response[a] *T. pyriformis* Vitamin Assay Media
Pantoyltaurine	10	33[b]	100	..
	1,000	..	..	95[b]
6-Aminonicotinamide	83	44[c]	..	..
	50	..	75	27[c]
Pyrimethamine	1.0	0[e]	20	..
	5.0	..	..	9[d]
Isoniazid	25	..	100	..
	25	..	..	24[e]

[a] Growth is expressed as a % of that of the control, e.g., (absorbance of test culture/absorbance of control culture) × 100. [b] Pantothenic acid assay. [c] Niacin assay. [d] Folic acid assay. [e] Pyridoxal assay.

nistic to folic acid in the *L. casei* system (3) it did not seem to affect the folic acid assay with *L. leichmannii*. It has been suggested that primidone and phenobarbital may antagonize folic acid in man (2), and Spray (9) reported that phenobarbital stimulated growth of *L. leichmannii* in vitamin B_{12}-deficient media. In the present studies no effect of either primidone or phenobarbital was evident. In tests with *T. pyriformis* chloroquine phosphate, chlorpromazine, methantheline bromide, methapyrilene hydrochloride, and promazine appeared to inhibit growth in vitamin B_6-deficient media. As reported previously (6) chlorpropamide, metahexamide, tolbutamide, and propylthiouracil interfered with the pantothenic acid assay with *T. pyriformis*. Phenformin frequently stimulated growth, but the effect was not specific for any vitamin assay; similar stimulatory effects at subinhibitory concentrations of toxic compounds have been reported (10).

Microscopical examination of *T. pyriformis* cells grown with each of the drugs shown in the lower section of Table III revealed differences in size, in granulation, and motility of cells grown with different drugs. Somewhat similar changes were noted however in control cultures at various stages of incubation from one to seventy-two hours. Any drug altering the growth rate might be expected to produce apparent changes in cell size, granulation, or motility, and therefore the significance of the differences observed was not evident.

Studies of Possible Drug-Vitamin Interrelationships.—The possible relationship between phenformin and folic acid, suggested by the initial screening test with *L. leichmannii*, was examined and growth response curves to folic acid with several concentrations of phenformin are shown in Fig. 2. The data strongly suggest an antagonism similar to that reported for proguanil hydrochloride (3) with *L. casei*. Although the inhibition due to diphenhydramine in folic acid-deficient media was slight, the inhibitory effect appeared to be real; growth responses (per cent of standard response) in the presence of 83, 100, and 200 mcg./ml. of the drug were 85, 85, and 80, respectively. A similar effect was not observed with *T. pyriformis* but Sanders and Nathan (11) reported that the toxicity of diphenhydramine for this protozoan was partially reversed by histidine.

The interrelationships between drugs and vitamins with *T. pyriformis* were also studied and data for some drugs are given in Figs. 3 and 4. There appeared to be a marked interrelationship between the oral antidiabetic drugs chlorpropamide, metahexamide, and tolbutamide and calcium pantothenate; propylthiouracil seemed to have a similar "antipantothenate effect." Although the magnitude of the effects of drugs exhibiting apparent anti-B_6 acitivity was not as large as for the sulfonylurea drugs, the effects also are probably real as can be seen in Fig. 4. Other workers (12, 13) have also indicated a relationship between chlorpromazine and pyridoxal.

Although growth of *T. pyriformis* was not quite complete after an incubation period of seventy-two hours, a three-day incubation period was used in both the toxicity studies (maximum noninhibitory concentrations of drugs) and in the screening tests

TABLE III.—THE EFFECT OF SOME DRUGS ON VITAMIN ASSAYS WITH *L. leichmannii* AND *T. pyriformis*

Drug	mcg./ml.	Vitamin Limiting[a] None	Niacin	Pantothenic Acid	Folic Acid	Vitamin B_{12}	Thiamine	Riboflavin	Pyridoxal
Tests with *L. leichmannii*									
Diphenhydramine	83	101	99	100	*85*[b]	100	...	...	...
Phenformin	83	100	102	98	*55*	100	...	...	...
Phenobarbital	50	98	101	100	93	96	...	...	...
Primidone	83	100	100	100	97	100	...	...	...
Proguanil hydrochloride	16.5	97	97	92	102	98	...	...	...
Tests with *T. pyriformis*									
Chloroquine phosphate	15	97	100	132	93	..	100	97	*33*
Chlorpromazine	1.25	99	102	104	94	..	97	86	*50*
Chlorpropamide	100	100	86	*11*	91	..	93	92	*77*
Metahexamide	100	90	100	*11*	100	..	88	100	81
Methantheline bromide	100	120	104	100	..	..	86	116	*21*
Methapyrilene hydrochloride	25	99	87	100	98	..	80	81	*30*
Phenformin	10	106	104	145	133	..	102	117	92
Promazine	1.25	97	103	106	98	..	94	86	*64*
Propylthiouracil	25	97	107	*51*	94	..	95	89	100
Tolbutamide	50	94	86	*11*	91	..	100	83	85

[a] See footnote[a] Table II. [b] The error was small in tests with *L. leichmannii* and small apparent inhibitions were rechecked.

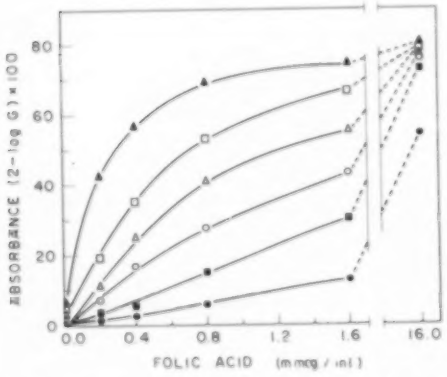

Fig. 2.—The effects of several concentrations of phenformin on the growth response of *L. leichmannii* to folic acid. The concentrations of drug in mcg. per ml. were: 0 (▲——▲), 25 (□——□), 50 (△——△), 100 (○——○), 200 (■——■), and 400 (●——●).

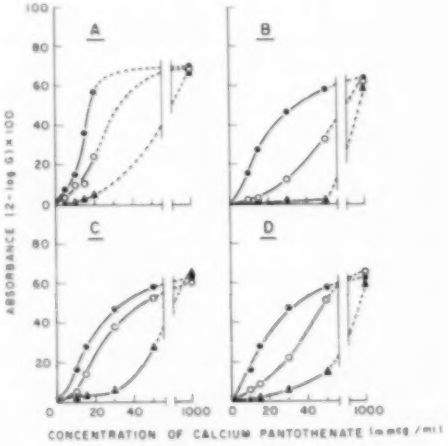

Fig. 3.—The effects of several drugs on the response of *T. pyriformis* to calcium pantothenate. The uppermost curves (●——●) are for the normal growth response. *A*, Propylthiouracil, 30 mcg. per ml. (○——○) and 50 mcg. per ml. (▲——▲). *B*, Metahexamide, 50 mcg. per ml. (○——○) and 200 mcg. per ml. (▲——▲). *C*, Chlorpropamide, 40 mcg. per ml. (○——○) and 125 mcg. per ml. (▲——▲). *D*, Tolbutamide, 30 mcg. per ml. (○——○) and 150 mcg. per ml. (▲——▲).

for antivitamin effects. It was of interest, therefore, to examine some of the drug-vitamin interrelationships after forty-eight and ninety-six hours' incubation; results are shown in Table IV. The data suggest that the inhibitory effect of a given concentration of drug decreased as the incubation time was increased or as the concentration of the appropriate vitamin was increased. The four drugs examined in this way appeared to reduce the rate of growth of the protozoan particularly during the first forty-eight hours of incubation with suboptimal concentrations of the vitamin.

In view of the results with the sulfonylurea drugs it seemed likely that the sulfa drugs might also exhibit "antipantothenate activity." In preliminary tests with several of the common sulfa drugs there was no suggestion of an antipantothenate action; only four of 50 drugs tested exhibited this effect. In higher animals, propylthiouracil and the oral sulfonylureas have some similar actions; propylthiouracil may produce hypoglycemia (14, 15) and some of the sulfonylureas have an antithyroid action (16). Winters, *et al.* (17), reported that pantothenate-deficient rats showed increased sensitivity to insulin. It has been reported (18) also that carbutamide and tolbutamide inhibit a coenzyme A-acetylating system *in vitro*. Boshell, *et al.* (19), reported that tolbutamide inhibits ketogenesis and have sug-

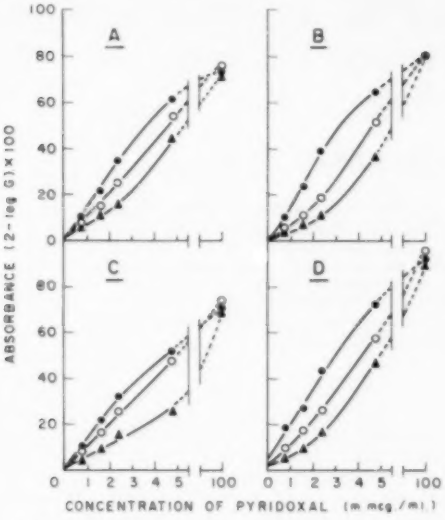

Fig. 4.—The effects of several drugs on the growth of *T. pyriformis* to pyridoxal. The uppermost growth curves (●——●) are the normal growth response curves. *A*, Promazine, 0.5 mcg. per ml. (○——○) and 2.0 mcg. per ml. (▲——▲). *B*, Methapyrilene hydrochloride, 5.0 mcg. per ml. (○——○) and 20.0 mcg. per ml. (▲——▲). *C*, Chlorpromazine, 0.5 mcg. per ml. (○——○) and 2.0 mcg. per ml. (▲——▲). *D*, Methantheline bromide, 100 mcg. per ml. (○——○) and 200 mcg. per ml. (▲——▲).

gested that this drug interferes with CoA metabolism. It seems likely therefore that the hypoglycemic action of the sulfonylurea drugs may be due to interference with some aspect of pantothenic acid metabolism.

It is not clear how much significance can be attached to the results of microbiological screening tests since the incidence of "false positive" results (5) is unknown. It is obvious too that microbiological tests cannot detect altered vitamin requirements of animals due to diuresis, altered absorption, or to changes in the intestinal flora. Certainly confirmatory tests must be done with higher animals before making any suggestion that a drug may (or may not) have an antivitamin effect in man. Aside from the possible practical nature of these findings in nutrition, however, such drug-vitamin studies may help elucidate the mechanism of action of certain drugs.

TABLE IV.—THE EFFECT OF INCUBATION TIME ON SOME APPARENT DRUG-VITAMIN INTERRELATIONSHIPS IN *T. pyriformis*

Drug	mcg./ml.	Concn. of Test Vitamin mmcg./ml.	Effect of Drug on Response at Three Incubation Periods[a] 48 hr.	72 hr.	96 hr.
Promazine	2	1.6[b]	60	55	70
		2.4	50	65	77
		4.8	38	85	92
		100	58	100	97
Methapyrilene hydrochloride	20	1.6[b]	36	39	46
		2.4	30	35	54
		4.8	38	73	87
		100	82	100	100
Propylthiouracil	50	10[c]	34	43	42
		15	42	24	42
		20	37	29	54
		1,000	97	96	100
Metahexamide	50	10[c]	19	37	49
		15	18	37	41
		30	18	33	52
		50	25	51	55
		1,000	99	100	100

[a] See footnote[a] Table II. [b] Pyridoxal. [c] Calcium pantothenate.

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Effects of Physostigmine on the Egg-Day Survivals of Chloramphenicol-Treated Embryonated Chick Eggs

By VERNON A. GREEN and JOHN E. DAVIS

Increases in egg-day survival were used as a criterion for determining potentiation, by physostigmine, of the antibacterial activity of chloramphenicol on bacteria-infected embryonated chicken eggs. Per cent egg-day survivals were increased by physostigmine potentiation of 0.1 mg./egg of the antibiotic. Attempts to enhance the antibacterial activity of higher doses of chloramphenicol resulted in a decrease in the survival percentage, probably due to the toxicity of the antibiotic. Both experimental observations were interpreted as being due to an increase in the penetration of the antibiotic into the chick embryo.

PHYSOSTIGMINE-induced increases in permeability of some tissues to certain chemicals have been reported—Barbour (1), Lewis (2), and Greig (3,4). Green, *et al.* (5), reported potentiation of the antibacterial activity of streptomycin and a reversal of the antibiotic activity of penicillin by physostigmine *in vitro*.

In this work, possible physostigmine potentiation of the antibacterial activity of chloramphenicol in embryonated chick eggs infected with *E. coli* or *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) was studied by the technique used by McKenna and Taylor (6) in growing bacteria cultures in yolk sacs. The criterion for determining potentiation was an increase in the per cent of egg-day survival above the egg-day survival of the eggs receiving the antibiotic alone.

EXPERIMENTAL

E. coli-Infected Eggs.—Chicken eggs were incubated at 37° for five days, after which they were candled; those eggs having living embryos were retained for the study. As the eggs were candled the positions of the air sac and the blood vessels of the chorio-allantoic membrane were marked on the shell with a wax pencil. After cleaning the shell with 70% alcohol, a dent was then made in the shell above the air sac and above the blood vessels, being very careful not to rupture the membranes; and using aseptic technique, 0.2 ml. of a suspension of *E. coli*[1] was injected, through the dent above the air sac, deep into the yolk sac. After sealing the dent through which the injection was made with paraffin, the eggs were returned to the incubator at 37°. Six hours after the inoculation of the eggs with bacteria, the eggs were again candled and those in which the embryos had died were discarded. Those having living embryos were divided into groups containing 34, 26, 10, 10, 10, 10, 32, and 46 eggs each. These groups were then given injections of 0.2 ml. of various mixtures, as indicated, through the membrane over the blood vessels: 34 eggs, 0.1 mg./egg of chloramphenicol,[2] 26 eggs, 0.1 mg./egg of chloramphenicol and 0.0015 mg. of physostigmine salicylate; 10 eggs, 0.2 mg./egg of chloramphenicol; 10 eggs, 0.2 mg./egg of chloramphenicol and 0.0015 mg. of physostigmine salicylate; 10 eggs, 0.4 mg./egg of chloramphenicol; 10 eggs, 0.4 mg./egg of chloramphenicol and 0.0015 mg. of physostigmine salicylate; 32 eggs, 0.8% sterile saline; and 46 eggs, 0.0015 mg./egg of physostigmine salicylate (see Table I). The holes in the shells were then sealed with paraffin and the eggs returned to the incubator and maintained at 37°.

After seven hours the eggs were again candled, and thereafter candled twice daily at 9 a. m. and 4 p. m. The egg-day survivals were determined daily for fourteen days, at which time any surviving embryos were destroyed, and the total-day survivals for each group recorded. The average survival time per egg was calculated and, using the average egg-day survival of the saline controls as 100%, the average survival time of each group was reported as a percentage of the egg-day survivals of the controls.

Micrococcus pyogenes var. Aureus-Infected Eggs.—Using the method described, the physostigmine salicylate potentiation of the antibacterial activity of chloramphenicol in embryonated chick eggs infected with *Staphylococcus aureus* was studied. The dose of the bacterial suspension was 0.2 ml.[3] injected deep into the yolk sac. After six hours at 37° the surviving embryos were noted, and the eggs divided into groups of 10, 13, 14, 15, 22, and 15. Each group then received over the blood vessels 0.2 ml. of a solution containing the following: 10 eggs, 0.1 mg./egg of chloramphenicol; 13 eggs,

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[1] The *E. coli* suspension was made from organisms grown in beef heart infusion, concentrated, washed twice, and resuspended in sterile saline. The suspension contained 312,000 organisms per ml. as determined by a plate count.

[2] The chloramphenicol as Chloromycetin, courtesy Parke-Davis and Co., Detroit, Mich. Chloromycetin solution ampuls, 0.5 Gm. in 2 cc., were used in this study.

[3] Micrococcus suspension was prepared by growing the organism in beef heart infusion, concentrating the organism, washing twice, and resuspending in sterile saline. The suspension contained 540,000 organisms as determined by the plate count method.

TABLE I.—POTENTIATION OF CHLORAMPHENICOL ACTIVITY IN *E. coli*-INFECTED[a] EMBRYONATED CHICK EGGS BY PHYSOSTIGMINE

	No. of Eggs	Egg-Day Survivals	Survival, %
0.1 mg./Egg chloramphenicol	34	36	240
0.1 mg./Egg chloramphenicol and 0.0015 mg./egg physostigmine salicylate	26	95	900
0.2 mg./Egg chloramphenicol	10	14	350
0.2 mg./Egg chloramphenicol and 0.0015 mg./egg physostigmine salicylate	10	18	440
0.4 mg./Egg chloramphenicol	10	19	470
0.4 mg./Egg chloramphenicol and 0.0015 mg./egg physostigmine salicylate	10	12	300
0.0015 mg./Egg physostigmine salicylate	46	25	130
Controls 0.2 ml. of 0.8% sterile saline	32	13	100

[a] Injection of 0.2 ml. of *E. coli* suspension containing 312,000 organisms per ml. was used to infect eggs.

0.1 mg. egg of chloramphenicol and 0.0015 mg./egg of physostigmine salicylate; 14 eggs, 0.15 mg./egg of chloramphenicol and 0.0015 mg./egg of physostigmine salicylate; 22 eggs, 0.0015 mg./egg of physostigmine; and 15 eggs, 0.8% sterile saline (see Table II). They were then returned to the incubator at 37°.

After seven hours the eggs were again candled and thereafter were candled twice daily at 9 a. m. and 4 p. m. The embryo survivals were reported as a percentage of the egg-day survivals of the saline injected controls.

TABLE II.—POTENTIATION OF CHLORAMPHENICOL ACTIVITY IN *Micrococcus pyogenes*-INFECTED[a] EMBRYONATED CHICK EGGS BY PHYSOSTIGMINE

	No. of Eggs	Egg-Day Survivals	Survival, %
0.1 mg./Egg chloramphenicol	10	18	390
0.1 mg./Egg chloramphenicol and 0.0015 mg./egg physostigmine salicylate	13	38	630
0.15 mg./Egg chloramphenicol	14	28	430
0.15 mg./Egg chloramphenicol and 0.0015 mg./egg physostigmine salicylate	15	32	460
0.0015 mg./Egg physostigmine salicylate	22	28	270
Controls 0.2 ml. 0.8% sterile saline	15	7	100

[a] Injections of 0.2 ml. of *Staphylococcus aureus* suspension, containing 540,000 organisms per ml. were used to infect eggs.

RESULTS

In this study of chloramphenicol potentiation by physostigmine in *E. coli*-infected eggs, it was noted that the egg-day survival of those eggs receiving 0.1 mg. per egg of chloramphenicol and physostigmine had 9 times the egg-day survivals of the controls while eggs receiving 0.4 mg. of chloramphenicol had an egg-day survival of 4.7 times the controls (see Table 1). There was also noted an increase in the egg-day survival of eggs receiving 0.1 mg. of chloramphenicol and physostigmine over the survival of those eggs receiving 0.1 mg. of chloramphenicol alone, an increase of 660%.

The potentiation of chloramphenicol activity in staphylococcus-infected eggs was not as dramatic as the effect on eggs infected with *E. coli* in this study; however, the eggs receiving 0.1 mg./egg of chloramphenicol and physostigmine had egg-day survivals that were 6.3 times that of the controls but only 1.6 times the survivals of those eggs receiving 0.1 mg./egg of chloramphenicol alone (see Table II). Eggs receiving 0.15 mg./egg of chloramphenicol alone had only about 40% more egg-day survivals than those receiving 0.1 mg./egg of chloramphenicol alone, and had less egg-day survivals than those with 0.1 mg. chloramphenicol plus physostigmine.

DISCUSSION

It would appear from the data in Table I that physostigmine potentiation rendered 0.1 mg. of chloramphenicol more active in inhibiting *E. coli* than 0.4 mg. of chloramphenicol alone or in combination with physostigmine. In fact, by extrapolation one might arrive at the conclusion that physostigmine potentiation of 0.1 mg. of chloramphenicol on *E. coli* in eggs was of a magnitude that should be equivalent to the antibacterial activity of 0.8 mg./egg of chloramphenicol alone. This would be a fallacy since these authors found, in preliminary investigation to determine the dose of chloramphenicol to be used in this study, a consistent decrease in per cent egg-day survivals of noninfected embryonated chick eggs with 0.5 mg. per egg of chloramphenicol (see Table III). Thus the observed decreases in egg-day survivals with doses of chlor-

TABLE III.—EFFECTS OF CHLORAMPHENICOL ON EGG-DAY SURVIVALS OF NONINFECTED EMBRYONATED CHICK EGGS

Dose of Chloramphenicol, mg./Egg	No. of Eggs Used	Total Egg-Day Survivals, day	Egg-Day Survivals, %
0	10	138	100
0.05	10	139	100+
0.1	10	137	99+
0.2	10	139	100+
0.3	10	136	98+
0.4	10	131	94+
0.5	10	119	86+
0.6	10	121	87+
0.7	10	101	73+
0.8	10	88	63+
0.9	10	67	49+
1.0	10	43	31+

amphenicol higher than 0.1 mg., when potentiated by physostigmine salicylate, may have been due to the toxicity of chloramphenicol.

In staphylococcus-infected eggs, it was also noted that physostigmine potentiation of 0.15 mg. of chloramphenicol gave less egg-day survivals than the 0.1 mg./egg of chloramphenicol potentiated by physostigmine. Here again, the toxicity of chloramphenicol is a probable factor.

The bacteriostatic activity of salicylates on some bacteria, and especially on *S. aureus* is well recognized (7). In this study, had there been a summation of the bacteriostatic activity of physostigmine salicylate and the antibiotic activity of chloramphenicol, the per cent egg-day survival for *S. aureus*-infected eggs receiving physostigmine and 0.15 mg. of the antibiotic, would have been approximately 700%; and that of *E. coli*-infected eggs receiving physostigmine and 0.2 mg. of chloramphenicol about 480 %. However, it was found that physostigmine salicylate with 0.2 mg. of chloramphenicol in *E. coli*-infected eggs and 0.15 mg. of chloramphenicol in *S. aureus*-infected eggs resulted in per cent egg-day survivals less than the per cent egg-day survivals of physostigmine and 0.1 mg. of the antibiotic in infected eggs.

Increases in the permeability of mouse brain and dog erythrocytes were reported by Greig, *et al.* (3,4). Further, Green, *et al.* (5), reported a 45% potentiation of the antibacterial activity of streptomycin on *E. coli* by physostigmine. In this investigation it appears that physostigmine salicylate possibly increases the permeability of the tissues of the chick embryo to chloramphenicol. Decreases in per cent egg-day survivals on attempts to potentiate doses of the antibiotic above 0.1 mg./egg may be the result of an increase in the tissue concentration of the antibiotic, which is of such magnitude as to be toxic.

Green, *et al.* (5), reported a decrease in chloramphenicol bacterial growth-inhibitory activity *in vitro* with physostigmine, but this finding was later found to be the result of a dilution-solubility factor. In the study on which the report was based, it was found that the solvent properties of the compound solvent for chloramphenicol were nullified by dilution so that microscopic crystals of chloramphenicol formed and could be brought down by centrifuging.

Although this particular technique of egg sac bacterial culturing has its limitations and is considered by some to be an obscure if not a poor method, it proved to be quite satisfactory for the purpose of this study. It afforded a convenient method of studying the effects of cholinesterase inhibition in a biological system lying intermediate between bacteria and mammals.

SUMMARY

In this study the antibacterial activity of chloramphenicol in *E. coli*-infected eggs was greatly potentiated by physostigmine when the dose of the antibiotic was low, as indicated by an increase in egg-day survivals. Chloramphenicol 0.1 mg./egg gave an egg-day survival 2.3 times that of controls, while 0.1 mg./egg of chloramphenicol plus physostigmine gave a survival 9 times that of the controls.

The attempts to potentiate higher doses of chloramphenicol resulted in less increase in egg-day survivals; in fact, an attempt to potentiate 0.4 mg./egg of chloramphenicol with physostigmine in *E. coli*-infected eggs resulted in a decrease in egg-day survival below the egg-day survival chloramphenicol 0.4 mg./egg alone.

Increases in egg-day survivals brought about by 0.1 mg./egg of chloramphenicol and physostigmine over chloramphenicol 0.1 mg./egg alone in staphylococcus-infected eggs were not as marked as those in *E. coli*-infected eggs. Nevertheless, there was a potentiation of some magnitude, and a decrease in egg-day survivals with higher doses of chloramphenicol when combined with physostigmine. Thus, not only was there a potentiation of the antibacterial activity, but there appeared to be a potentiation of the toxicity of the antibiotic which may have been due to an increased tissue concentration of the chloramphenicol.

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Drying of Tablet Granulations

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A discussion of tablet granulation drying theories, techniques, and available equipment is presented with particular emphasis on rotary vacuum drying methods.

PRECISION of dosage, durability, stability, appearance, and simplicity of administration are all features which recommend the tablet to patient, physician, and manufacturer as a satisfactory dosage form. Although tablets have become the most widely employed mode of administering drugs orally, the predominant techniques for their preparation have not changed significantly over the past years. For the vast majority of tablets the standard or classic method for preparing granulations, that is, the final form of the mixed solids most suitable for use in the tablet machine, consists of six steps as illustrated in Fig. 1.

The addition of liquids during the preparation of a granulation permits the formation of granules. With proper control of the drying phase of the operation an optimum residual moisture content, a necessity in high speed tablet compression, is obtained. Reduction of the static charge on the granulation due to the presence of a suitable quantity of moisture often prevents bridging in the hoppers and feed frames during compression, with the added advantage of providing better interparticulate bonding.

Unlike chemical manufacturing where moisture is present as the result of precipitation or washing techniques, tablet granulations contain moisture deliberately added to a dry mixture in order to obtain specific operational advantages. It is important to emphasize this since it is a key point that granulations are not dried until they are moisture free. For each type of tablet press and feed frame, optimum quantities of residual moisture are dependent on the physical characteristics of the particular solids and will, therefore, vary from one formula to another.

The necessary residual moisture in the finished tablet granulation has generally been obtained by drying on trays, using warmed air as the carrier medium for the moisture which is vaporized. In this report we have endeavored to study the various types of equipment available for the drying of tablet granulations with reference to their relative merits in light of current theoretical concepts covering the drying of granular materials. Particular emphasis has been placed on the employment of rotary vacuum drying techniques.

THEORY AND FUNDAMENTAL CONCEPTS OF DRYING OF SOLIDS

Though the composition of individual tablet granulations may vary considerably, the fundamental mechanisms governing the drying of the granulations remain constant for any given dryer setup. Drying, which shall be defined here as the removal of a liquid from a solid by thermal means, is controlled by a well-defined set of external and internal variables (1). The length of time required for drying as well as the characteristics of the finished granulation will depend on the inherent physical attributes of the completed granule and the specific dryer employed, both of which will be described in greater detail in the following paragraphs.

Most studies of the drying of granular materials have concerned themselves with single chemical entities which were homogeneous throughout. In contrast to this, tablet granulations are complex systems consisting of many separate ingredients bonded together as a result of the granulation process. The nature of the internal mechanisms controlling drying of this material are, however, quite similar to those of single component systems. New designs and improvements in current techniques of drying must necessarily be based on sound theoretical grounds. It will be advantageous to review briefly these fundamental principles of drying operations as a basis for the subsequent detailed discussion of drying methods and equipment.

Without a temperature gradient to evaporate the liquid from the surface of the solid, drying cannot proceed. The rate of movement of liquid

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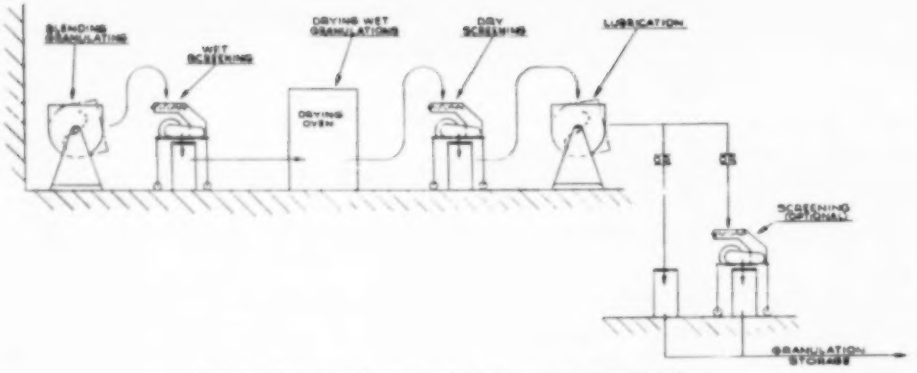

Fig. 1.—Standard 6-step method of preparing granulations.

from the interior of the solid is dependent on the rate of surface evaporation, the temperature of the material, its texture and structure, its water content, and the way in which the moisture occurs throughout the material. The drying operation itself may depend upon heat transfer by convection, conduction, radiation, or some combinations of these. However, regardless of the mode of heat transfer, the heat usually contacts the outer surface first, followed by transmission to the interior of the solid. The single exception is drying with high frequency emission which generates heat internally and hence produces a higher temperature within the solid than on its surface.

The determination of how a solid dries depends upon the effect of external conditions and on the internal mechanism of fluid flow. The external conditions such as temperature, humidity, air flow, and state of subdivision are all easily-measurable factors, and thus drying rates are usually based on these values. Further, the results so obtained are more or less directly applicable to the establishment of the design of dryers. Since these external mechanisms will later be discussed extensively in conjunction with the effective operational characteristics of dryers, a closer look at the internal mechanisms controlling drying will be taken at this time.

Although the internal mechanisms are of lesser practical significance, their study remains of great interest. Drying processes within beds of solid particles are concerned with liquid flow, vapor flow, and heat transfer. Depending upon the structure of the solid, internal liquid flow may occur by one or a combination of several postulated methods. Some of the mechanisms include (2–4): (*a*) capillary flow in granular and porous solids, (*b*) diffusion in homogeneous solids, (*c*) flow caused by pressure gradients, (*d*) flow caused by gravity, and (*e*) flow caused by a vaporization-condensation sequence.

Though all of the above-mentioned processes are in operation under certain circumstances, the first two mechanisms predominate during the drying of pharmaceutical granulations and are worthy of further elaboration.

As noted by Comings and Sherwood (5) in their studies of the drying of granular solids, the capillaries are nonuniform, being neither circular nor straight, but water nevertheless is pulled through the passages in a manner similar to the movement of water in a tapered capillary tube. This movement of liquid water through a solid undergoing drying is illustrated by the following experiment. A glass tube was drawn out to form a tapered capillary and filled with water. It was then allowed to remain exposed to the air until the water had completely evaporated (Fig. 2). It was noted that the meniscus remained stationery at the small end of the tube while the other meniscus moved progressively from the large toward the small end, until all the water had been evaporated.

Fig. 2.—Vaporization of water from a tapered capillary.

In loose granular beds, the small force provided by the surface menisci exerts sufficient capillary pull to draw liquid through any passage ending in a larger cross section. Because of the intricate passageways beneath the surface, it is possible for the replacement air to enter readily, and thus the moisture concentration near the surface can remain relatively high. In tightly packed wet granular material with no possibility of air replacement the force that is developed produces only a slight suction compared to that produced at the bottom surface (6). Therefore, normal capillary flow cannot carry the liquid to the surface and a more complicated situation arises.

As the drying proceeds, a time will be reached when the capillary action cannot maintain sufficient suction at the surface and water will no longer be drawn to it. Evaporation will still continue at the surface menisci, but soon the water in these surface openings will be depleted and its level will retreat into the solid. The rate of drying will consequently be slowed down and vapor diffusion will then become the principle mode of water loss. The temperature differential within the system then increases in importance since a force is required to

diffuse the vapor through the passages to the surface for removal. Several authors (7, 8) have substantiated the presumption that drying takes place from water surfaces in the interior of the solid.

Thus on the basis of internal mechanisms alone, one could summarize the process of air drying of porous granular solids as follows: as drying proceeds, capillary forces acting on the liquid in the bed tend to draw the liquid to the drying surface against the action of gravity and friction. In the initial phases, the drying rate will be constant since the surface will be sufficiently saturated to maintain the air film at the interface in a saturated condition.

However, this condition will be modified as the surface saturation falls below a critical value at a point referred to as the critical moisture content. The drying rate then diminishes in a regular fashion until the entire surface layer is dry, after which liquid removal takes place by internal vapor diffusion through the now open capillaries to the surface. Capillary movement of liquid will take place in the granules as well as in the spaces between the granules. Since the granule pore diameters would, in general, be considerably smaller than the surrounding capillaries, the bulk of the free liquid will be removed before the pore liquid. Discontinuous movements of moisture such as this are another factor contributing to the changes in shape observed in drying rate curves.

The drying cycle for all solids may be divided into three distinct zones: a heating period, a constant rate of evaporation period, and a falling rate of evaporation period. The heating period is of minor importance since it is usually quite short in comparison with the total drying time. The drying zones can best be illustrated by the use of drying rate curves shown in Fig. 3. These rate curves show that the drying process is not a smooth continuous one controlled by a single mechanism. Section *MN* on each curve represents the constant rate period of drying while *NP* is termed the falling rate period. It will be noted that the rate of drying is changing continuously throughout this second interval. The point *N* on each curve denotes the critical moisture content.

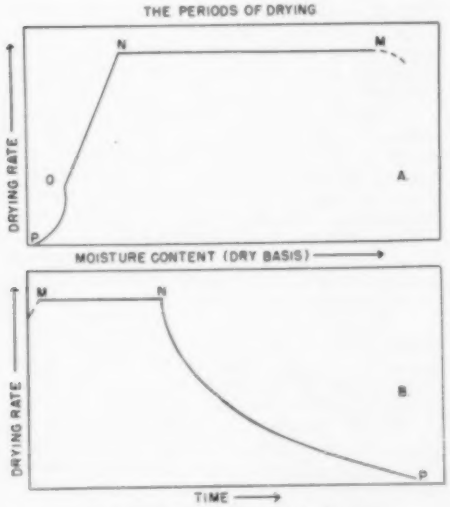

Fig. 3.—The periods of drying.

Constant Rate Period.—During the constant rate drying period, the moisture content of the material is above the critical value. The rate of evaporation is essentially independent of the solid since the rate approaches the condition existing with a layer of liquid with no solid present. The vapor pressure at the surface of the solid will be equal to the vapor pressure of the liquid at the temperature of the surface. The rate of drying is determined by (*a*) the rate of diffusion of vapor through the air film at the surface of the solid, (*b*) the moisture content and relative velocity of the drying air, and (*c*) the surface temperature of the material.

Thus, when the drying material is made to contact hot surfaces with frequent agitation in such systems as tumbler dryers, drying rates are usually greater than under corresponding conditions in trays. As long as the above factors remain unchanged, the drying rate will be constant until the moisture content at the surface reaches its critical level.

Falling Rate Period.—At the critical point the rate of drying will start to decrease continuously. The drying interval which follows is then designated as the falling rate period. There are two distinct zones found within this period: the zone of unsaturated surface drying, and the zone where internal fluid flow controls drying rate.

The first zone, immediately following the critical point, is frequently characterized by a linear relation between the rate of drying and the water content (9). The second zone may be characterized by a nonlinear curve as noted in Fig. 3 *B*. The definition between the two zones of the falling rate period may vary considerably from the ideal conditions as set forth above. In most pharmaceutical granulations the zone controlled by internal fluid flow predominates in determining the ultimate drying time.

It is possible however, even with the same material, to vary the conditions so that the falling rate curve may be wholly linear, completely nonlinear, or include a portion of both zones of falling rate drying (9). When a material to be dried shows a linear falling rate, it becomes obvious that surface evaporation is the controlling factor in the process and that the rate of drying can be varied by changing such external conditions as air velocity, humidity, and temperature. On the other hand, with materials in which the internal resistance to moisture movement is controlling the drying, changes in the external drying conditions will not change the rate of evaporation from the surface. Rather, the rate of drying will be increased by reducing the thickness of the material, by increasing the temperature within the granule, and/or by reducing the external pressure. The more significant factors responsible for controlling internal liquid flow have been set forth previously as diffusion, capillarity, and pressure gradients.

The constant rate phase of drying has presented

relatively few difficulties as to its mechanism. The falling rate phase, just described, exhibits complex mechanisms which have not yet been fully unraveled. Several current theories (9–11) have as their basis various physical concepts which explain the phenomenon in a more exact mathematical fashion. However, vapor diffusion and capillary flow do not lend themselves readily to precise experimental verification.

An important factor related to external drying conditions is the condition of the surface of evaporation. In certain types of granulations the initial drying rate should be slowed down to prevent too rapid drying of the surface material. With rapid drying, surface shrinkage and case hardening result, thereby slowing down the subsequent drying rate. This is a largely unexplored area in tablet granulation drying as precise studies of optimum conditions of temperature for drying have never been reported.

Vacuum Drying.—Prior to this point, the discussion has been concerned with the theories of atmospheric drying. During the past decade many investigations have contributed to the theory of air drying with relatively little work reported on drying under vacuum. A great majority of the same basic premises are thought to apply to this type of drying, but experimental evidence supporting the theories (12, 13) is quite sparse.

The first stage of vacuum drying is presumed to be a constant rate period (7). As the surface water is removed rapidly, movement of moisture continues by means of capillarity and vapor diffusion. Available water, under conditions of vacuum, means any water that is free to vaporize, regardless of its position in the bed. This concept achieves more importance since during vacuum drying a greater amount of water may evaporate internally and diffuse to the surface as vapor. Moisture lost in this way results in modified physical characteristics of the dried solid, some of which are quite valuable in pharmaceutical granulation processing. The specific advantages imparted to the granulation will be discussed in a later section.

Comprehensive data on vacuum drying of granulations are not available at this time and, as a result, many questions concerning drying techniques remain unanswered. Information yet to be clarified includes: (*a*) optimum vacuum required for drying of the various pharmaceutical ingredients, (*b*) nature of moisture movement within granules under the influence of vacuum, (*c*) methods of obtaining maximum heat transfer under evacuated conditions in enclosed systems, (*d*) effects of air bleeds on drying rates in vacuum systems, (*e*) water vapor permeability of surface of granules as drying proceeds, and (*f*) relationship between rate at which heat is transferred to the material and rate at which the vapors are removed.

Although the above constitute only a few selected problems in the area of drying under reduced pressures, they definitely point to the great need for more extensive research in this area by pharmaceutical investigators. Researches in vacuum technology are now under way in our laboratories in an attempt to obtain information of value to workers in the field of tableting.

FACTORS DETERMINING THE SELECTION OF A DRYER

There are in existence a great variety of machines known as dryers, many of which might possibly be applied in drying pharmaceutical granulations. There are, however, many unique requirements in the pharmaceutical industry which tend to rule out most of the configurations and principles which are so widely applied in other industries. It is the purpose of this section to discuss some of the pharmaceutical considerations which must be evaluated when selecting drying equipment.

One of the prime considerations in drying granulations is the relatively small batch size involved. This tends to eliminate most types of continuous drying equipment on two counts. Most continuous equipment is both fairly large and also relatively difficult to clean. The large size necessitates high capital investment. The difficulty involved in complete cleaning between batches introduces the problem of high labor costs. Therefore, continuous dryers are impractical for use in most cases and are only economically justifiable in the largest volume products.

The requirement for easy cleaning has the attendant consideration of low product retention. The small quantity, high value ratio inherent in pharmaceuticals makes it important to have an absolute minimum amount of material lost in cleaning. This factor would again eliminate most continuous dryers and many batch dryers with internal baffles or pockets which would tend to collect portions of the granulation being dried.

Material handling is an important aspect of drying processes. High labor costs are involved in manipulating the material by hand, as in shelf and tray dryers. Capital investment cost and additional cleaning problems are associated with the use of conveyors, chutes, adapters, and the like which are often incorporated with either continuous dryers or automated batch dryers.

In addition to the above physical characteristics of the dryer itself, there are many chemical and physical requirements of the granulations and the drugs which they contain. The trend in drugs seems to be toward more potent and reactive chemicals which are frequently susceptible to oxidation and decomposition. Therefore, anything which can be done to decrease the possibility of chemical reactions taking place during drying will make the dryer that much more acceptable. To decrease the possibility of oxidation as well as other types of decomposition, low temperature and the exclusion of oxygen are both important.

The problems of instability can be approached from several directions. One method is the substitution in air dryers of an inert gas such as nitrogen, a change which is prohibitively expensive. An alternate is to use reduced temperature air drying, an operation usually too lengthy to be practical. A logical solution is the use of vacuum drying which solves the problem from both approaches. In the first place, it excludes air from the drying chamber. Secondly, it permits the granulating solvents to be vaporized at a much lower temperature or at a much higher rate with the same temperature. A contingent advantage is the ease of solvent recovery, where desirable.

Other problems involved in drying granulations are related to the subsequent processes which they must undergo. As stated in the introduction, the granulation must not be completely dry but should contain a uniform, optimum residual moisture content which will vary from one type of granulation to another.

Optimum moisture content is believed to be necessary for the maintenance of the various granulation ingredients, particularly gums, in a hydrated state. If these ingredients are over-dried their properties are sometimes altered and subsequent rewetting will not always return them to their former hydrated state. Another advantage of the presence of residual moisture is the reduction of static electric charges on the granules. Such charges can exert significant and disturbing effects during the compression of tablets. Therefore, the drying process must be readily controlled so that the moisture content of the finished granulation can be varied from product to product. Uniform moisture content is required for satisfactory control of the drying process. It can be achieved in several ways including blending, comminuting, or aging in closed containers, but is obtained most simply in a moving bed dryer.

An optimum particle size range for each granulation is required to facilitate flow to the dies of the compressing machine, for proper disintegration of the tablet, and for the control of various physical properties of the tablet. Therefore, as with moisture content, there is also an optimum particle size distribution, which is usually obtained by grinding after drying. However, if the drying process causes too much attrition, there will be too many fines in the finished tablet granulations.

In solving any problems of drying, all of the above points must be considered and the advantages must be weighed against the disadvantages. The following section is a discussion of many of the various types of commercially available dryers which are used in the tablet sections of the pharmaceutical industry. Included is a short discussion of some of the more obvious advantages and disadvantages of each type of dryer.

Tray and Shelf Dryers.—Tray drying has been used extensively in drying granulations for many years. Due to changing economic conditions and the development of more potent and sensitive drugs, these dryers are gradually being replaced, at least in some plants. A factor of economic importance is that tray dryers require a great amount of manual labor to spread the wet granulation on paper lined trays and then empty the trays into a comminuting machine after the granulation is dry. A second and more critical difficulty with tray drying is that the drying medium is heated air, even for materials prone to oxidation. A third disadvantage is that tray drying is quite slow, often requiring twenty-four to thirty-six hours of drying prior to the attainment of the optimum residual moisture content.

The advantages of this type of dryer are low cost, simplicity of construction, ease of mechanical operation, and absence of cleaning required to accommodate product changes. Vacuum shelf dryers have been used to counter some of the disadvantages of conventional shelf dryers, but these dryers are considerably more expensive and still have the disadvantage of high labor cost and poor control of uniformity of moisture remaining in the granulation.

Fluid Bed Dryers.—The fluid bed dryer is an air agitation type of batch dryer in which the granulation is dried by means of passing a heated air stream through the batch from the bottom. The granulation is thereby maintained in a fluid state. Losses are minimized by collecting any particles which might be blown out of the system in collector bags. The rapidity with which drying is achieved (less than an hour in most cases) keeps the granulation's contact time with heat and air at a minimum. It is claimed that cleaning of the collector bags and hopper can be done rapidly and with ease. Although the time interval of drying is greatly reduced, the effective total volume of air required for fluidizing the material is probably equal to or greater than the quantity of air employed in tray drying. An attendent disadvantage stems from the amount of product retention within the unit if changeover is required very often. On the positive side is the reduced amount of material handling when compared with conventional tray drying and, of course, the extremely short drying cycle.

Turbo Dryer.—The turbo or rotating shelf dryer is another type of dryer which has been used with varying degrees of success in the pharmaceutical industry. The rotating shelf dryer is essentially a continuous dryer which circulates hot air over a granulation being fed from tray to tray by a cleaning arm and a spreading arm. The granulation is fed in wet at the top and comes out dry at the bottom.

The advantage of this type of dryer is the moving bed which allows uniform drying to optimum moisture with a minimum of attrition, as compared to many other types of moving bed dryers. The turbo dryer might be very useful on a large volume product where a control system could be developed. However, for small quantities of granulation, batch control would be most difficult as would be cleaning for changeovers. Also, the limitations of high temperatures and subjection to contact with oxygen would be present. Therefore, even for a high volume specialty this dryer would add little except speed and automated materials handling to the drying cycle.

Radiation Dryers.—Usually, radiation dryers are of the infrared variety. They have been used effectively in a few instances in the pharmaceutical industry. It would seem from the investigations of Fowler (14) that the development work required to adapt this type of dryer would rather limit its use to high volume products because of the many variables involved; e. g., wavelength required, time, surface temperature, air stream temperature, bed depths, and method of agitation.

An advantage of this type of dryer would be a shorter drying cycle resulting in less exposure time to the high temperatures involved in drying. Another advantage might be a somewhat higher thermal efficiency which is of questionable value in the small scale drying of pharmaceuticals. One of the drawbacks inherent to radiation heating processes is that heat is transferred only to surfaces which are "seen" by the radiating source, and thus the surface of the particles tends to overheat. Unless the layer is extremely thin, the result will be that only the top surface of the layer will dry.

The use of this type of drying equipment, especially under vacuum, might be of value and worthy of development work for large production items. Its use in conjunction with vacuum drying would benefit heat transfer which is usually limited to conduction alone.

Dielectric Dryers.—The dielectric dryer is actually a type of radiation dryer although heat is transferred by means of radio frequency emissions. The difference between this type of dryer and other radiation type dryers is that heat can be transmitted throughout the granules, not to the surface alone. This type of dryer is still somewhat of an oddity or "unusual demonstration" type with few industrial applications of dielectric drying being reported. Dielectric heating may be used for the evaporation of water from heat-sensitive organic solutions and slurries. A commercial application in the drying of pharmaceuticals and biologicals has been reported by Brown, *et al.* (15).

Ultrasonic Drying.—Although ultrasonics is a relatively expensive form of energy, it may eventually find application in the drying of heat-sensitive products or chemicals which have very long drying cycles. It is theoretically possible to apply acoustic drying to almost any type of dryer suitable for the drying of granular materials. It has been suggested (16) that an excellent dryer system would use conventional heating to take out easily removed moisture and ultrasonics to remove the last percentages. Ultrasonics may be a tool to be applied to increase the effectiveness of any existing dryer by either reducing required drying temperatures or increasing the speed of end point drying.

Spray Dryer.—Spray drying is usually reserved for products which are in a liquid or slurry form. In general, the spray dryer is useful for specific ingredients, but because of the liquid feed stock it is impractical for use in making a granulation.

Pneumatic Dryers.—The pneumatic dryer configuration is somewhat of a cross between the fluidized bed dryer and the spray dryer. A falling granulation is dried by a rising stream of heated air. This dryer can be constructed with a single or multistage design. A dryer of this general type used in preparing granulations and in coating tablets has been described by Wurster (17). This type of dryer would have the same drawbacks as the fluidized bed dryer; however, it does show some promise as a new tool in the area of granulating and coating technology.

Rotating Dryers.—There are many types of rotating dryers so that it is difficult to treat them all as a single subject. On the other hand, there are so many different types of rotary dryers which utilize many of the same principals that it is likewise difficult to separate them all into unique categories. Rotary dryers are applicable to either batch or continuous process, some designs being used for both types of drying.

The advantages of a rotating dryer are as follows: (*a*) a continuously changing surface is presented to both the drying medium and the heating medium; (*b*) attrition can be adjusted so that desired particle size is maintained and build-up or agglomeration kept at a minimum by selecting speed of rotation; (*c*) where the tendency for agglomeration is pronounced, internal baffles or paddles can be put into the dryer chamber to break up lumps; (*d*) the construction of a rotary dryer lends itself to simplicity in cleaning and maintenance; (*e*) almost any range of heating temperature can be attained from a red heat, as in kilns, to room temperature, as in an unjacketed dryer, or moderate heat or cold in a jacketed dryer; (*f*) in continuous drying, time of exposure to drying media can be controlled so that almost any degree of dryness can be achieved; and (*g*) most types of rotary dryers are adaptable to vacuum drying.

There are many different types of rotary dryers but those most acceptable to the pharmaceutical industry are either the double cone dryer or the "pair of pants" dryer (otherwise known as the "V" dryer). Both of these types of dryers provide a folding action which is gentle but thorough and can be accomplished without internal contrivances. The uncluttered innards make these dryers easy to wash and dry completely in a very short time to provide the fast turn around and versatility required by the pharmaceutical industry.

ROTARY VACUUM DRYING SYSTEM[1]

Except for the jacket and vacuum connections this dryer is similar in appearance and in mixing action to the cone blender. Since minimal materials handling, instrumentation leading to automation, and suitable mixing action were potential advantages, our investigation centered about this type of equipment. A wide range of operating capacity for any given size Rovac dryer makes it useful over a fairly large segment of batch sizes. The absence of internal baffles or pockets permits quick and easy cleaning with small loss of material when changing from one product to another. Materials handling is minimized by loading the dryer from above and emptying from below directly into a comminuting machine.

Opportunities for degradation of the ingredients during the drying cycle are minimized because of the exclusion of oxygen and the use of lower drying temperatures made possible by drying in a vacuum. A greater uniformity of residual moisture content is achieved because of the moving bed, and by controlling within fairly narrow limits the length of time and temperature of the drying cycle. Once the above information is established, operating conditions are duplicated in an almost automatic fashion. Particle size distribution can be controlled by varying the speed of rotation and drying temperature as well as by comminution of the dried granulation.

As a result of the excellent potential utility this apparatus offered, a small (8.8 cu. ft.) capacity dryer (Fig. 4) was designed and purchased. Experimental studies on various granulations with this unit demonstrated that with proper cycles, rapid drying of granulations could be achieved. In addition, the mobile state of the solids during drying resulted in granulations with superior particle size distribution and surface characteristics. These granulations ran well on the high speed rotary presses and yielded satisfactory tablets from the quality point of view. Following eight months of intensive developmental research it was felt that the utilization of Rovac drying was feasible for

[1] Rovac is the abbreviated name applied to the rotary vacuum drying system as designed by Ciba.

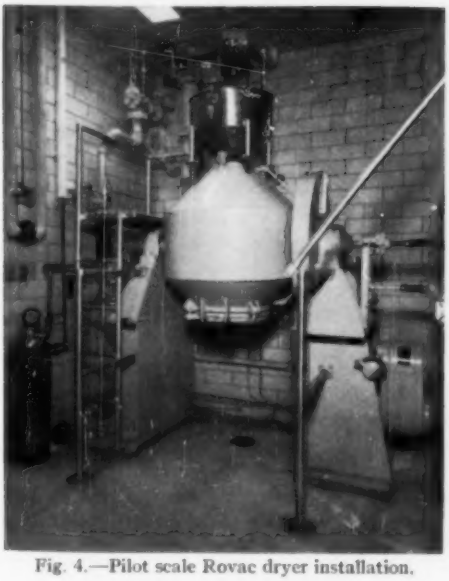

Fig. 4.—Pilot scale Rovac dryer installation.

Fig. 5—Overall view of Rovac dryer installation in Ciba tablet granulation section.

production scale operations. It was therefore decided to incorporate the Rovac dryers (Fig. 5) into the design of a new granulating department, but the existing tray dryers were included for use until correct Rovac cycles for all tablet granulations could be established. It was determined that the size of the dryer itself should be such that the majority of batches fill it to 50 to 60% of internal volume. The versatility of this dryer however permits operation with almost any quantity of material as long as there is enough room for the tumbling action to be effective. For this reason a machine with a total capacity of 24 cu. ft. and an average working capacity of 12-14 cu. ft. was designed. It was decided that the dryer should have a variable speed rotational drive in order to provide the flexibility needed to ascertain optimal operating speeds for batch sizes beyond those possible on the smaller experimental unit. Since a 5:1 ratio drive motor was available and it was felt that speeds much higher than 20 r.p.m. would result in insufficient movement of the granular bed within the dryer because of centrifugal forces, the speed range selected was 4 to 20 r.p.m.

Vacuum System.—It was found during the development work that the highest vacuum economically obtainable was to be preferred. Mechanical vacuum pumps were used exclusively during this phase and the highest vacuum obtained was about 29 inches of mercury. This vacuum was found to decrease drying cycles from about eighteen hours for tray dryers at similar temperatures to less than four hours in the Rovac dryer. In actuality, a vacuum of about 29.5 inches of mercury is obtained in currently operating equipment which improves the drying time to about two to three and one-half hours for most standard granulations. Since the vacuum system must handle as many as eight tumblers simultaneously, the design features required special attention. The vacuum apparatus was established as two individual steam jet systems. One system is employed for continuous operation in carrying out the actual drying and is designated as the "hold-down" system. The other vacuum system, designated the "draw-down system" is used to create a vacuum within individual dryers prior to the dryer being switched over to the "hold-down" system. This was necessary in order to prevent other dryers already on "hold down" from being "shocked" when a new one is added to the system. The use of two systems therefore reduces any chance of reverse flow in the vacuum lines which would carry dust from previous batches back to material currently being dried. Vapors from one batch are also prevented from flowing into another batch. The "draw down" vacuum was sized to evacuate the 24 cu. ft.-dryer from atmospheric pressure to 29 inches of mercury in five minutes. To properly operate the eight dryers simultaneously, the "hold down" vacuum was sized to handle 35 pounds per hour of 50% ethanol-50% water mixture plus 10 pounds per hour of air leakage, at 29 inches of mercury.

Jacket Temperature Control System.—Although vacuum drying can be conducted at ambient temperatures the availability of controlled elevated temperatures permits the development of abbreviated cycles and broadens the utility of the equipment. There are several methods available to supply the heat required for the evaporation of the granulating liquid, but the one chosen was a jacketed dryer utilizing water as the heating and cooling medium. In order to obtain the widest temperature control and reduce corrosion in the jackets, a completely closed system operating with either hot or chilled water was used. By means of three-way valves a temperature controller admits water at either 60° or 10° to make up heat gains or losses in the system. The range of heating can be expanded to include a range of from 5 to 90° should this be required at any time in the future. The control system modulates the addition of either hot or cold water to maintain the desired temperature. With the use of a proportioning electric temperature controller and a three-way motorized valve capable of proportional control, there is little difficulty in maintaining

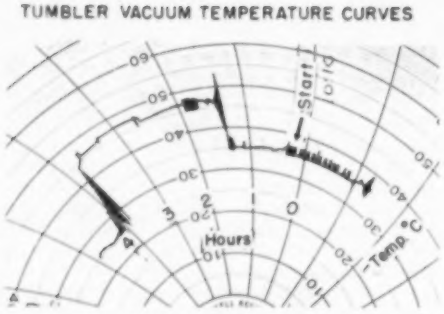

Fig. 6.—Recording of temperature in dryer jacket during a dual temperature drying cycle.

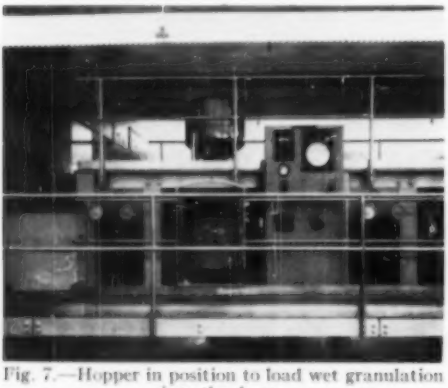

Fig. 7.—Hopper in position to load wet granulation into the dryer.

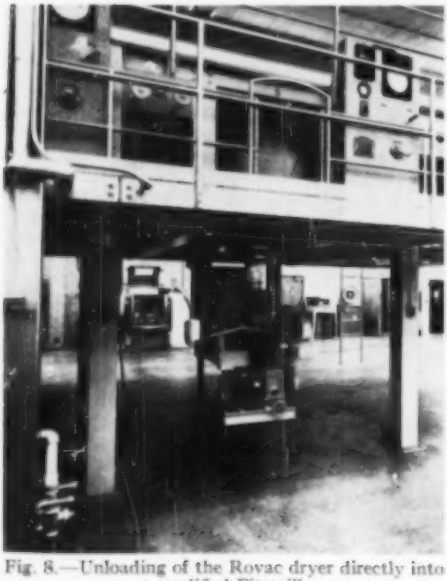

Fig. 8.—Unloading of the Rovac dryer directly into a modified Fitzmill.

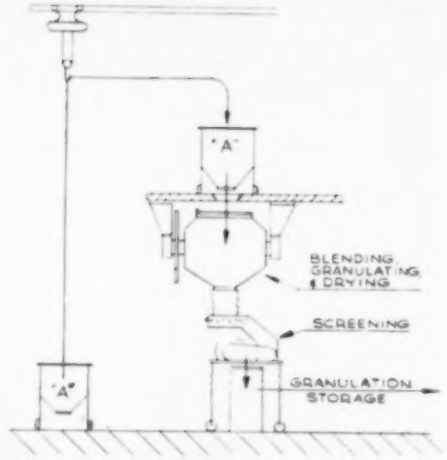

Fig. 9.—Flow sheet for vapor method of preparation of self-lubricating granulations.

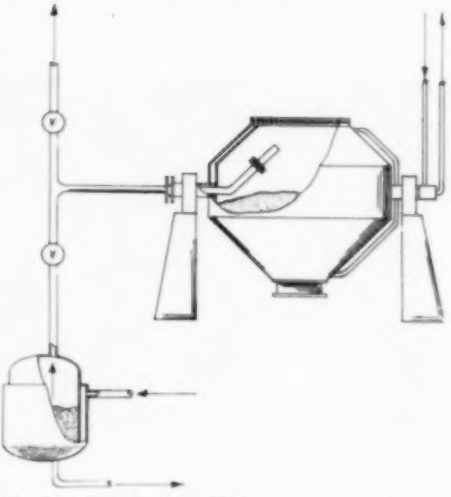

Fig. 10.—Drawing from U. S. patent covering vapor granulation technique.

limits of ±2° simultaneously on as many as eight different dryers, each operating at a different temperature. Figure 6 is a recording of the temperature in the tumbler jacket during a dual temperature drying cycle.

General Operation of the Rovac System.—A view of the hopper in position to load the granulation into the dryer is shown in Fig. 7. To the left of the dryer is the vacuum panel body with both the "draw-down" and the "hold-down" vacuum system controllers. Also present is a duplex vacuum gauge with two indicator hands. One hand senses the pressure on the "hold down" system; the other senses the pressure in the dryer. When both hands read similarly, the dryer may be switched from the "draw-down" to the "hold-down" system. The

main operating panel for the dryer is found to the right of the unit. In the upper left corner is the indicating temperature controller for jacket input water and just below, a mercury-filled thermometer for jacket outlet water temperature. The recording thermometer in the upper right-hand corner derives readings from the temperature controller. Various selector switches for both the electrical and mechanical controlling of the unit fill out the rest of the panel. Figure 8 shows the dryer unloading directly into a modified Fitzmill.

From a labor time point of view, the cleaning of the tumblers presented the greatest problem, particularly since the system is used for many products of varying composition and color. An effective solution resulted from the development of a special cleaning apparatus which makes it possible to wash the interior surfaces with a high pressure hot water spray and to drain without entering the dryer. The washing cycle takes fifteen to twenty minutes with little operator attention required.

Since the double cone tumbler represents an adequate device for blending dry powders, the elimination of the separate wet granulating step seemed to be the next logical direction in which to move. If successful, the flow sheet for preparing tablet granulations would approximate the procedure outlined in Fig. 9 Investigations are currently in progress employing this adaptation of the Rovac dryers to permit one-step granulating. In a series of experiments in our laboratories it has been shown that granulating solids with vaporized liquids yielded extremely uniform granulations. Thus, material wetted with a vaporized granulating solution and subsequently dried under vacuum was removed from the Rovac as a finished granulation ready for compression. Under such circumstances the entire granulating process covered a time interval of less than four hours. A drawing, from a U. S. patent (18) covering the above-mentioned process illustrated how this is accomplished (Fig. 10).

CONCLUSIONS

The development and regular utilization of Rovac drying in our plant did not represent the simple adaptation of a vacuum dryer to pharmaceutical purposes. In addition to the codevelopment of self-lubricated granulations (19), other modifications, and analysis of material handling costs, the most significant aspect of the project was the re-education of the research, engineering, and production personnel in the basic principles of pharmaceutical process engineering as it concerns the manufacture of tablet granulations. A similar approach in other areas of pharmaceutical manufacturing might prove to be equally valuable.

A careful analysis of the present state of pharmaceutical technology reveals that a great deal of the equipment used is representative of types developed primarily for the chemical, bakery, and other industries. In addition, much of the processing is imitative and lacking in specificity for the dosage forms which constitute the end products of our industry. This situation results from the weak position of process development in pharmaceutical manufacturing operations, the strong traditionalism in pharmacy, and the absence of a machine industry geared specifically to supply the changing needs of the pharmaceutical manufacturer.

The drying of tablet granulations represents only one phase of the problem of manufacturing quality tablets economically and it is obvious from this paper that a great deal of developmental research remains to be accomplished before an ideal system is devised and put into practice. In view of the relative importance of the tablet in modern therapy, there is no doubt but that the cost in time and money of such research would be well rewarded.

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Spray-Drying of Tablet Granulations I

A Preliminary Report

By A. M. RAFF, M. J. ROBINSON, and E. V. SVEDRES

A preliminary report describing a new tablet granulation is presented. The process for preparing tablets using this granulation is described. Potential advantages resulting from its use are discussed in the following areas: (*a*) ease of manufacture, (*b*) improvement of stability, (*c*) utilization of pigment colorants, (*d*) admixture of incompatible chemicals, (*e*) tableting of thermolabile and moisture-labile materials, and (*f*) improvement in tablet physical properties.

THIS PAPER is a preliminary report of a new basic tablet granulation and a rapid and inexpensive process for preparing tablets using this granulation.

Until now, commercial tablet manufacture was based on one of two standard methods, wet granulating or slugging (double compression). These methods are lengthy, expensive, dusty, and inflexible in that the granulations must usually be specially formulated for each different active ingredient. In addition, conventionally prepared granulations are often the cause of such difficulties as the following in tablet manufacture: capping, caused by an excess of "conventional" fines in the granulation; the incorporation of thermolabile or moisture-labile materials; mottling (spotting) during coloring of the tablets; batch-to-batch color variation; and excessive moisture content of the finished granulation, leading to short shelf life with moisture-sensitive products.

Granulations prepared by the methods described in this paper have none of these undesirable characteristics. It is possible to prepare a granulation rapidly and inexpensively that has a uniform distribution of particle size within a very narrow range. This granulation then may simply be admixed with any active ingredient. This type of granulation allows the production of tablets having excellent color distribution.

This method is much more rapid because the following steps of conventional wet granulating are eliminated: reduction of particle size, preparation of granulating solution, granulating, screening of wet mass, drying, and screening of dry material.

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The free-flowing granules obtained from this procedure of preparing granulations, having a uniform distribution within a very narrow particle size range, allow better control of weight and size of compressed tablets and eliminate capping and chipping.

DESCRIPTION OF PROCESS

A slurry is prepared containing a filler (excipient), disintegrant, and binder in a liquid vehicle. When a colored basic granulation is desired, a coloring agent may be added to this slurry. The slurry is then spray dried to form dry particles comprising the filler and disintegrant, substantially coated with the binder. The spray drying is carried out in a conventional apparatus. The resultant material is then a finished granulation which is mixed with an active medicinal ingredient and, when necessary, a lubricant. The mixture is then compressed on a tablet press.

Specifically, the initial step of preparing the granulation consists of measuring the liquid vehicle into a vessel of the proper size. Using a mixer, the binding agent is dissolved in the liquid vehicle. To this solution the disintegrant is first added, and then the filler. Throughout the operation, as each additional ingredient is added, the slurry must be continuously agitated in order to prevent settling of the heavier particles.

The slurry is then fed into a spray dryer. The outlet temperature will be within the range of 25° below the boiling point of the liquid vehicle to about 25° above. The inlet temperature will be higher than the outlet temperature; possibly as high as the limitations of the equipment will permit. During the course of the operation the inlet and outlet temperatures remain substantially constant. The dried granulation is collected in a receiver at the bottom of the main chamber.

The solids concentration of the slurry will normally be from 50 to about 60%, by weight.

The liquid vehicle used in preparing the slurry or suspension should be low boiling, having a boiling point from about 35 to about 150°. Examples are water, ethyl alcohol, carbon tetrachloride, and chloroform.

The filler or excipient can be any used in industrial pharmacy. The filler is present from about 50 to about 98%, by weight, of the total solids of the slurry described above. Some of the more common fillers are kaolin, lactose, magnesium carbonate, sucrose, and calcium sulfate dihydrate.

The binders used will vary from about 2 to 10%, by weight, of the total solids. Binders used are natural gums and gum constituents such as acacia, tragacanth, sodium alginate, agar, etc. Cellulose

esters such as methyl or ethylcellulose, carboxymethylcellulose, hydroxymethylcellulose, and proteinaceous materials such as gelatin, casein, and zein may also be used.

The disintegrating agent is present from about 3 to 20%, by weight, of the total solids. The material used as a disintegrating agent is any natural or synthetic water-insoluble, water-swelling material such as alginic acid, starch, flour, or acid CMC.

Magnesium stearate, talc, stearic acid, etc., may be used when a lubricant is required.

The granulation is composed of substantially spherical dry granules comprising a filler and disintegrant coated with a binder.

The particle size distribution of the granulation, when prepared in a (Nerco-Niro) laboratory size spray dryer, ranges from about 10 to 70 μ (Sharples Micromerograph analysis). While the same particle size range (10 to 70 μ) may be achieved in a production size unit (Nerco-Niro 7 feet, 6 inches diameter), it was found more advantageous commercially to prepare the material in the particle size range of approximately 100 to 250 μ. The larger size granulation was found to possess the same advantages as the smaller size laboratory material, and further could be prepared at a maximum production rate.

The finished granulation is formed by mixing the desired powdered medicament with the basic granulation and a lubricant, such as magnesium stearate The granulation may now be compressed on any standard tablet press.

The ratio of medicament to basic granulation may vary from 1:1 to 3:1. The upper limit of the per cent weight of medicament in the finished tablet is critical and, in most cases, will not exceed 90% of the finished tablet weight. The lower limit is less critical and may be as low as necessary.

The most advantageous ratio of the basic tablet granulation to active ingredients varies widely depending upon the physical characteristics of the active ingredients, i. e., granular, fluffy, amorphous nonflowable material, amorphous free-flowing material, crystalline material not readily compressed, or crystalline material readily compressed.

EXPERIMENTAL

An example of this process, carried out in the laboratory, is described below:

Formula of Slurry

Calcium sulfate dihydrate	41.25% w/w
Alginic acid	7.00
Acacia, U. S. P., powdered	1.50
Guar gum	0.25
Water	50.00

Preparation of Slurry.—The water was placed in a stainless steel container. Using a propeller type mixer, the guar gum was added with constant agitation and mixed until a thin gel formed. The acacia was then added and mixing continued until it had dissolved. Lastly, the alginic acid and calcium sulfate dihydrate were added and mixing continued until a uniform slurry resulted.

Spray Drying of Slurry.—Using a concurrent, centrifugal wheel atomizer (Nerco-Niro laboratory model) spray dryer, the dryer was equilibrated with the inlet temperature at 225° and the outlet temperature at 110°. Spray-drying of the slurry was commenced at the rate of 60 Gm. of water per minute with the outlet temperature being maintained by regulating the amount of slurry being pumped to the atomizing wheel. The atomizing wheel revolved at a rate of 40,000 r. p. m. The dried granulation, consisting of substantially spherical granules of calcium sulfate dihydrate, and alginic acid coated with acacia, were collected in the chamber below the cyclone of the unit. The particle size distribution of the granulation, as measured on the Sharples Micromerograph, was from about 10 to 70 μ (see Table I).

TABLE I.—PARTICLE SIZE DISTRIBUTION ANALYSIS

Diameter of Particle Size Range, μ	Percentage Weight Within Range
60–65[a]	10
55–60	10
50–55	8
45–50	8
40–45	8
35–40	8
30–35	8
25–30	9
20–25	9
15–20	9
10–15	8
5–10	5

[a] Approximately a No. 230 N. B. S. screen.

Preparation of Tablets

Ingredients	mg./Tablet
Chlorpromazine hydrochloride U. S. P.	5.00
Magnesium stearate U. S. P.	1.00
FD&C Yellow No. 5 aluminum lake[1]	1.35
FD&C Blue No. 1 aluminum lake	0.07
Spray dried granulation (as prepared above)	162.00

All of the ingredients except the spray dried granulation were passed through a No. 60 U. S. Standard screen and mixed well for ten minutes. The spray dried granulation was added and mixed for an additional ten minutes. The mixture was then compressed on a Stokes BB-2 using $^{9}/_{32}$ inch flat faced, bevel edge punches.

DISCUSSION

Preparing a slurry of the ingredients is very advantageous in that a much more homogeneous mixture is obtained. This not only affords ideal mixing of the ingredients but also is most advantageous when a colored tablet is desired.

In this case the dyes, pigments, or lakes are mixed in a liquid state with the other ingredients and spray dried. Since the material is instantly dried there is no chance for migration of the colorant, and uniform color distribution results.

If pigments or lakes (insoluble dyes) are to be

[1] The authors wish to thank National Aniline Division of Allied Chemicals for their generous supply of FD&C aluminum lakes.

used as a variation of the process they can be mixed with an uncolored granulation and, because of the fine particle size of the granulation, exceptional color distribution is obtained. This overcomes the disturbing problems of mottling and batch-to-batch color variation. This uniformity is illustrated in Fig. 1. Shown are two rows of tablet slugs. The upper row is composed of tablets made by mixing a standard granulation with two percentages of black pigment and then compressing the mixture. The lower row is composed of tablets using the basic granulation.

Two further variations that are afforded by this process with regard to color are possible. Using this spray dried basic granulation and FD&C aluminum lakes one can prepare color triturations of the three primary colors, red, blue, and yellow and by suitable admixtures of these obtain any desired color. The color uniformity that results is primarily due to the ultrafine granulation produced by this process. This color uniformity could allow one to film-coat (1) tablets with a clear solution thereby eliminating another potential source of difficulty in the manufacture of colored coated tablets.

Comparison of two properties of the basic granulation and a standard granulation were made. The particle size distribution of the basic granulation is illustrated in Table I and Fig. 2. An indication of the degree of free-flow of the basic granulation can be gained by comparing its angle of repose[1] 27° [determined by the fixed funnel method (2)], with that of a standard wet granulation, 36°. The bulk volume (3) serves as an additional measure of the disparity between the two granulations. The basic granulation has a bulk volume of 1.36 ml./Gm. and the standard granulation has a bulk volume of 1.05 ml./Gm.

Preliminary studies show improved stability of tablets made with spray dried granulation. More particularly, this improvement has been noted for tablets having moisture-sensitive ingredients. Karl

Fig. 1.—Uniformity of mixing pigment colorants with dry granules.

[1] α = angle of repose = height of mound/radius of mound.

Fig. 2.—Angle of repose. Basic granulation on left, standard granulation on right.

Fischer determinations for moisture show as much as a 30% decrease in moisture content of the spray dried granulation *vs.* a dry-mix of raw materials normally low in moisture (i. e., about 1%). Comparative moisture studies of identical formulations of wet granulated *vs.* spray dried granulations have been made. Determinations by several methods have generally shown less moisture in the spray dried granulation, but the data has been erratic, to date, due to the degree of difference in particle size and surface area. Studies of tablets compressed from both type granulations and then similarly ground to uniform size showed about a 50% greater amount of moisture in the tablets made from wet-granulated material. Additional moisture studies are continuing.

Preliminary experiments indicate these granulations produce tablets with exceptional hardness and sheen. This surface characteristic permits a dust free tablet. We have been able to use less lubricant, e. g., as little as 0.05% of magnesium stearate, thus producing a more rapidly disintegrating tablet. A hard tablet may be produced using minimal pressure. This should result in much longer tool and tablet press life. Comparative studies will be reported in subsequent publications from this laboratory.

Finally, the use of this placebo tablet granulation leads to increased flexibility. It is possible to use the same granulation for many active medicinal ingredients. Tablet production is simplified because it is possible to shift from one tablet strength to another, or from one product to another on short notice. It is necessary only to add the new active ingredient to a standard granulation rather than to develop a new granulation.

SUMMARY

1. A method has been described for the manufacture of a basic tablet granulation, having a uniform distribution of particle sizes within a very narrow range, by spray drying a slurry comprising a filler, disintegrant, binder, and vehicle.

2. The advantages of such a granulation, in several areas, are discussed.

3. The use of pigment colorants in compressed tablets and their more convenient utilization in the manufacture of film-coated tablets is explored.

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Drug Standards

Properties and Analysis of Dextromoramide and Its Dosage Forms

By PAUL J. A. W. DEMOEN

Dextromoramide occurs as a white, microcrystalline powder which can be formulated for use as an analgesic in solutions for injection, compressed tablets, or suppositories. Dextromoramide base is practically insoluble in water but can easily be dissolved in dilute acids, giving stable solutions. The solid *d*-bitartrate salt is used to prepare dextromoramide formulations (solutions for injection, compressed tablets, suppositories). Dextromoramide can be determined in its dosage forms by several analytical procedures. The drug appears to be stable in its different formulations over long periods of time.

DEXTROMORAMIDE (*d*-2,2-diphenyl-3-methyl-4-morpholinobutyryl-pyrrolidine) is an analgesic substance which was synthetized some years ago (1), and whose pharmacological studies have shown that it is from 10 to 40 times more active than morphine in mice, rats, cats, guinea pigs, and dogs, under different experimental conditions (2–5).

Clinical reports confirmed the analgesic activity shown by the pharmacological experiments (6–20). Dextromoramide is therefore widely used as an anodyne (21). It is of particular interest because it may be used orally with almost the same effectiveness as the injections (6, 9, 14, 16, 17, 19, 20, 22–24).

Since habituation towards the analgesic effect is practically nonexistent, even after prolonged administration (6, 18, 19, 25, 26), dextromoramide is particularly indicated as an oral analgesic agent in the treatment of chronic pain, particularly in patients with cancer. Further indications are: intense pain in general, postoperative pain (11, 13, 14, 18, 27), obstetrical afterpains (24, 28–31), and in surgery, as an effective analgesic supplement to anesthesia (8, 25, 26, 32–37).

The side effects due to dextromoramide are comparable to those due to other potent analgesic drugs; therefore, it has been classified as a narcotic (38).

Although it appears to have no specific hypnotic properties (18, 19, 23, 32), dextromoramide markedly potentiates the hypnotic as well as the respiratory depressant action of barbiturates (8, 18, 25, 33, 39). In these cases, N-allylnormorphine is used as an antidote (8, 13, 18, 32, 40, 41). Dextromoramide has a relatively high therapeutic index (6, 8, 15) and low toxicity

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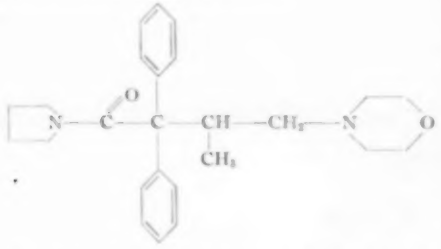
(8, 9). It may be administered in the form of parenteral injections, compressed tablets, or suppositories. The most suitable single dose is 5 mg. for injections (6, 7, 13, 15, 25, 32) and oral administrations (16, 19, 24), and 10 mg. for suppositories (9, 29, 30).

The onset of analgesic action is within five to fifteen minutes after subcutaneous or intramuscular injection (6, 9), and within ten to thirty minutes for the other dosage forms (9, 19), while the duration of the effect is between three and eight hours (6, 7, 9, 14, 16, 19, 42).

PHYSICAL PROPERTIES

Dextromoramide Base.—Dextromoramide base is a white, odorless, amorphous or microcrystalline powder, melting at 183.5–184.5°; empirical formula $C_{25}H_{32}N_2O_2$, molecular weight 392.52. It may be represented structurally as follows:



Dextromoramide base is practically insoluble in water and in petroleum ether. It is slightly soluble in diethyl ether, diisopropyl ether and hexane. One gram of it dissolves at room temperature in about 25 ml. of 0.1 *N* hydrochloric acid, 45 ml. of methanol, 50 ml. of ethanol, 30 ml. of isopropyl alcohol, 3 ml. of chloroform, 5 ml. of benzene, 50 ml. of acetone, or 35 ml. of ethyl acetate. Dextromoramide is a weak monovalent base with pK'a equal to 7.05 when measured in 50% methanol as described by Beckett and co-workers (43). It has the following specific rotation: $[\alpha]_D^{29} = +26 \pm 0.5°$ ($c = 5\%$ in benzene), $+16° \pm 0.5$ ($c = 5\%$ in ethanol), and $+30 \pm 0.5°$ ($c = 5\%$ in 2 *N* hydrochloric acid).

In slightly acid solution (0.01 *N* hydrochloric acid in 90% isopropyl alcohol) (44), dextromoramide shows an ultraviolet absorption band with the following characteristic points ($c \pm 0.1$ m*M* %): maxima at 256 mμ (355), 261.5 (385), 266.5 (305); minima at 253 mμ (345), 258.5 (340), 266 (305); approximate ε values are given in parentheses.

The infrared spectrum (2.5 to 15μ) of about one mg. dextromoramide in a potassium bromide disk shows absorption maxima at: 3.3–3.5μ (CH stretching), 6.1μ (carbonyl stretching of the amide C = O), 6.7μ (aromatic CH), 6.9μ (CH_2 and asymmetrical CH_3 vibrations), 7.2μ (symmetrical CH_3 vibration), 9.0μ (C—O stretching in aliphatic ether), 9.9 and 11.6μ (skeletal vibrations), 13.2 and 14.2μ (CH bending in monosubstituted benzene derivatives).

Dextromoramide gives definite, crystalline precipitates with many common alkaloid reagents, such as silicotungstic, phosphotungstic, phosphomolybdic, and picric acids (45). Like many alkaloids and synthetic analgesics (46–51), dextromoramide forms colored complexes with different color indicators (bromocresol purple, bromocresol green, methyl orange) in buffered aqueous media. These complexes have a great color intensity, and are extractable from the aqueous layer by means of an organic solvent, thus providing a method of quantitative analysis for relatively small ($\leq$30 mcg.) amounts of dextromoramide. This procedure (methyl orange) was used successfully in this laboratory to determine the total amount of dextromoramide in the urine of rats and dogs, following subcutaneous injection (52).

When chromatographed on buffered Whatman No. 1 paper using the ascending technique of Curry (53), dextromoramide gives an orange-brown spot after spraying with the modified Dragendorff reagent (54); R_f equals 0.84 ± 0.02.

Dextromoramide *d*-Bitartrate.—Dextromoramide *d*-bitartrate is a white, odorless, amorphous or crystalline powder, melting with slight decomposition at a temperature between 189 and 192°; empirical formula $C_{25}H_{32}N_2O_2 \cdot C_4H_6O_6$, molecular weight 542.61.

One gram of dextromoramide *d*-bitartrate dissolves at room temperature in about 20 ml. of water, 12 ml. of 0.1 *N* hydrochloric acid, 40 ml. of methanol, 100 ml. of ethanol, or 100 ml. of acetone. It is slightly soluble in isopropyl alcohol, diethyl ether, chloroform, or hexane, and insoluble in benzene, diisopropyl ether, and solutions of alkali hydroxides or ammonia.

Dextromoramide *d*-bitartrate has the following rotatory characteristics: $[\alpha]_D^{29} = +25.5 \pm 0.5°$ ($c = 5\%$ in water) and $+30.5 \pm 0.5°$ ($c = 5\%$ in methanol).

The infrared spectrum (2.5 to 15μ) of dextromoramide *d*-bitartrate in a potassium bromide disk shows intense absorption maxima at the following wavelengths: 3.0μ (OH stretching), 3.4μ (CH stretching), 3.8μ (broad band: amine salt), 5.95μ (carbonyl stretching of the acid), 6.3μ (carbonyl stretching of the amide), 7.2μ (symmetrical CH_3—vibration), 8.0μ (C—OH stretching in acid), 9.0μ (C—O stretching aliphatic ether), 9.3μ (C—OH stretching in secondary alcohol), 10.2 and 11.6μ (skeletal vibrations), 13.2 and 14.0μ (CH bending in monosubstituted benzene derivatives). The ultraviolet absorption characteristics of dextromoramide *d*-bitartrate are identical with those of the free base.

TYPICAL FORMULATIONS

Injection.—Solutions for subcutaneous or intramuscular injection containing either 5 or 10 mg. of dextromoramide (equivalent to 6.9 and 13.8 mg. of dextromoramide *d*-bitartrate) per ml. were prepared by dissolving the required amount of the *d*-bitartrate salt in water for injection. The solution was sterilized in an autoclave (twenty minutes at a pressure of one atmosphere); pH of the solution was 3.5. Isotonic solutions were prepared by adding 7.0 and 5.0 mg., respectively, of sodium chloride per ml., giving a freezing point of −0.54 to −0.57°.

Compressed Tablets.—Compressed tablets of dextromoramide were found to be stable when manufactured by the usual techniques (slugging or wet granulating). Tablets containing 5 mg. of dextromoramide (equivalent to 6.9 mg. of dextromoramide *d*-bitartrate), lactose, sucrose, and corn starch, and granulated with corn starch paste were prepared, having a disintegration time of three minutes at 37° in artificial gastric juice, and a hardness of about 4 Kg. (Stokes hardness tester).

Suppositories.—Since dextromoramide is rapidly absorbed from the organism, not only after oral administration or after injection, but also when administered rectally, dextromoramide suppositories containing 10 mg. of the product (equivalent to 13.8 mg. of dextromoramide *d*-bitartrate) in 1.3 Gm. of a neutral base (e. g., "adeps neutralis") were also prepared for purposes of this study. Melting point of the suppositories was 36 ± 1°.

Stability.—The stability of dextromoramide in its different dosage forms was tested at various storage temperatures over long periods of time. The results, summarized in Tables I, II, and III, show that dextromoramide exhibits good stability in all the formulations tested; indeed, no deterioration was observed in any dosage form, at room temperature (22 ± 2°), at 37°, or in the refrigerator (4 ± 1°) after a storage period up to three years.

TABLE I.—STABILITY OF DEXTROMORAMIDE IN SOLUTION FOR INJECTION CONTAINING 5 MG. PER ML.

Time	—Stability at Storage Temperature, %— 60°	37°	22 ± 2°	4 ± 1°
0	100	100	100	100
7 days	98	101	99	101
14 days	100	98	102	101
30 days	97	99	99	98
60 days	100	99	100	100
120 days	100	100	98	98
240 days	...	101	100	98
1 year	...	99	99	99
2 years	...	99	100	98
3 years	...	97	100	98

TABLE II.—STABILITY OF DEXTROMORAMIDE IN COMPRESSED TABLETS CONTAINING 5 MG.

Time	—Stability at Storage Temperature, %— 60°	37°	22 ± 2°	4 ± 1°
0	100	100	100	100
1 month	103	102	99	98
2 months	100	97	103	99
4 months	101	98	99	99
8 months	98	98	96	99
1 year	98	98	102	101
2 years	...	97	100	100
3 years	...	97	99	101

TABLE III.—STABILITY OF DEXTROMORAMIDE IN SUPPOSITORIES CONTAINING 10 MG.

Time.	Stability at Storage Temperature, % 22 ± 2°	4 ± 1°
0	100	100
1 month	100	98
2 months	97	100
4 months	100	98
8 months	102	99
1 year	102	98
2 years	98	102
3 years	99	100

ANALYTICAL PROCEDURES

Several analytical procedures can be used to determine dextromoramide in its dosage forms. In the absence of interfering substances, the product may be titrated nonaqueously with perchloric acid, or an ultraviolet spectrum may be measured after suitable dilution. In the presence of interfering substances, the base may be extracted and then determined either gravimetrically, or by one of the two procedures mentioned above.

Injection (5 mg. per ml.).—*Ultraviolet Spectrophotometric Method.*—Two milliliters (equivalent to 10 mg. of dextromoramide) of the solution is transferred to a 25-ml. volumetric flask, 0.5 ml. of 0.5 *N* hydrochloric acid is added, and the solution is diluted to 25 ml. with isopropyl alcohol. The dilution (about 40 mg. % of dextromoramide) is measured against a blank containing 10 ml. of 0.1 *N* hydrochloric acid diluted to 100 ml. with isopropyl alcohol. Maximum absorption is determined at 261.5 mμ. The concentration of dextromoramide is calculated using a molar extinction coefficient (ϵ) of 385.

Titration.—Ten milliliters (equivalent to 50 mg. of dextromoramide) of the solution is transferred to a 150-ml. beaker, and is evaporated just to dryness on a boiling water bath. The residue is dissolved by gentle warming in about 30 ml. of glacial acetic acid, and the solution is cooled. After the addition of 0.2 ml. of a 1% solution of α-naphtholbenzein in glacial acetic acid, the solution is titrated with 0.02 *N* perchloric acid in glacial acetic acid. One milliliter of 0.02 *N* perchloric acid corresponds to 7.850 mg. dextromoramide.

The presence of sodium chloride in the solution does not influence the results of the titration. By adding 2 ml. of a 2% solution of mercuric acetate in glacial acetic acid (55) after the end point has been reached, the amount of sodium chloride can be determined by titrating it with the same titrant to the next color change.

Injection (10 mg. per ml.).—Dextromoramide is determined by the methods already described, using 1 ml. of the solution for the spectrophotometric method or 5 ml. for the titration.

Compressed Tablets.—*Direct Titration.*—An amount of finely powdered tablets, corresponding to 50 mg. of dextromoramide, is transferred to a 150-ml. beaker. After the addition of 30 ml. glacial acetic acid, the mixture is stirred on a magnetic stirrer for five minutes. The mixture is titrated with 0.02 *N* perchloric acid in glacial acetic acid, using α-naphtholbenzein as an indicator. In place of a colored indicator, the titration may be followed potentiometrically (glass-calomel electrodes).

Gravimetric Procedure.—An amount of finely powdered tablets, corresponding to 200 mg. of dextromoramide, is transferred to a 50-ml. volumetric flask containing about 35 ml. of a 1% aqueous solution of tartaric acid. The mixture is shaken for thirty minutes, diluted to 50 ml. with 1% tartaric acid solution, and mixed. The suspension is filtered through a retentive filter paper.

Forty milliliters of the filtrate (equivalent to 160 mg. of dextromoramide) is transferred to a 150-ml. separatory funnel and shaken with 20, 15, and 15-ml. portions of ether. The ethereal layers are removed and discarded. The remaining solution in the separatory funnel is made alkaline with sodium hydroxide solution, and the liberated dextromoramide base is extracted with successive 20, 10, 10, 5, and 5-ml. portions of chloroform.

The chloroform is collected in a second separatory funnel, washed with 10 ml. of water, and filtered through a paper filter into a weighed evaporating dish.

The second separatory funnel and the filter are washed with two 5-ml. portions of chloroform which are added to the main fraction. The solution is evaporated on a water bath to about 5 ml. with the aid of a gentle current of dry air, 5 ml. of absolute ethanol is added, and the evaporation is continued just to dryness. The residue is moistened with 5 ml. absolute ethanol and evaporated again. The evaporating dish containing the pure dextromoramide base is dried for two hours at 100°, cooled, and weighed.

The purity of the residue may be determined by measuring its infrared or ultraviolet absorption spectrum, or by dissolving a weighed portion of it (about 60 mg.) in glacial acetic acid and titrating this solution with 0.02 *N* perchloric acid as described previously.

Suppositories.—*Direct Titration.*—Five suppositories are placed in a 150-ml. beaker and dissolved in 30 ml. glacial acetic acid. The solution is titrated potentiometrically as described above. A blank correction should be applied by performing a titration on an aliquot of the excipient.

SUMMARY

1. The physical properties of dextromoramide are described, along with methods of preparation of several common dosage forms.

2. Analytical procedures are developed for the assay of dextromoramide in these dosage forms.

3. Data are presented which demonstrate that dextromoramide in its different formulations is perfectly stable over long periods of time.

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Use of Ion Exchange Resins in the Adsorption and Elution of Association Colloids

By N. BRUDNEY

The anomalous adsorption and elution behavior of association colloids on ion exchange resins is described. Sodium ditertiarybutylnaphthalene sulfonate (DBNS) can be readily adsorbed onto almost any anion exchange resin if solutions below the critical micelle concentration (CMC) are used. This can be achieved either by the use of very weak aqueous solutions, or stronger alcohol/water solutions. DBNS cannot be eluted from resin adsorbates by aqueous eluants, but is readily removed by acidic alcohol solutions. Other association colloids show similar effects and these results are discussed from the point of view of association colloid solution structure. The results indicate that following normal adsorption, ions of the association colloid can be eluted by similarly charged ions. However, as soon as the concentration within the ion exchange resin particles reaches the CMC, the particle acts as a micellar sieve and the association colloid is not completely eluted.

THE advantages of ion exchange resin/drug complexes over the use of free drugs have recently been described (1). One of these advantages is that the resin adsorbate is tasteless and completely free of aftertaste. A material to which this technique has been applied recently was an association colloid, sodium ditertiarybutylnaphthalene sulfonate. It was found that the anionic portion of the molecule could be adsorbed, but that elution with aqueous solvents was not possible.

Another use of ion exchange resin adsorption of association colloids is in the field of shampoo analysis (2, 3, 4) and detergent analysis (5, 6). Here, too, it has been found that aqueous elution of ion exchange resin/association colloid complexes is not possible, and that alcoholic eluants are necessary.

Experimental results are described and an explanation of these effects is proposed.

EXPERIMENTAL

Sodium ditertiarybutylnaphthalene sulfonate (DBNS) was obtained from a commercial source[1] and was found to contain 9.33–9.42% sulfur (99.5–100.5% theoretical) and less than 0.1% inorganic sulfates, $E(1\%, 1\text{ cm.})$ on the Beckman DU spectrophotometer at 280 mμ = 165.0.

Sodium dodecyl benzene sulfonate (DDBS) was obtained from a commercial source[2] and was found to be relatively impure, containing 96.0% active ingredient, $E(1\%, 1\text{ cm.})$ at 260.5 mμ = 12.4.

All other materials used were of reagent grade where available. Deionized water was used, specific resistance more than 1.0 megohm cm.

Strong anion exchange resin Amberlite IRA 401, 7 Gm., (− 16 + 50 U. S. mesh) as received from the manufacturer[3] was washed well with water and formed into a 1 cm. diameter column in a glass tube. The resin was cycled through the hydroxyl form and chloride form and washed well with 2 L. deionized water. A 1% w/v solution (200 cc.) of DBNS was passed through the column at about 3 drops per minute and the effluent collected and made to 250 cc. This solution was diluted and the absorbance determined. By difference, it was found that 300 mg. had been adsorbed, whereas on a theoretical basis of meq./Gm. dry resin, the whole 2.0 Gm. should have been adsorbed.

Amberlite IRA 401, 250 mg., washed, cycled, rewashed and dried, was placed into each of 12 screw-capped glass tubes. Aqueous solutions of DBNS, 25 cc., of varying concentrations were added to each tube, and the tubes rotated for one hour at room temperature at such rate as to ensure that the resin was continuously suspended. The contents of each tube were separated and the amount of DBNS adsorbed determined spectrophotometrically by difference. This experiment was repeated at 40 ± 2°. The results are shown in Table I.

TABLE I.—THE ADSORPTION OF DBNS ON AMBERLITE IRA 401 USING AQUEOUS SOLUTIONS

Concn. DBNS, % w/v	% DBNS Adsorbed at 20°	% DBNS Adsorbed at 40°
0.001	83.0	68.0
0.003	94.2	93.0
0.005	97.5	94.7
0.007	96.7	96.9
0.009	97.0	97.4
0.01	97.5	96.0
0.02	87.3	97.5
0.03	68.5	87.0
0.04	45.5	57.3
0.05	38.9	37.0
0.06	27.3	28.5
0.07	24.0	22.5
0.08	25.5	23.7
0.09	21.5	19.2
0.1	12.8	15.2
1.0	1.0	5.5

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The author wishes to thank Mrs. H. Harder for her assistance with some of the spectrophotometric measurements.

[1] Société des Laboratoires Labaz, Brussels.
[2] Chemical Developments of Canada Ltd., Montreal.
[3] Rohm and Haas Co., Philadelphia, Pa.

It was apparent that adsorption was concentration dependent, and that below 0.01% w/v almost 100% of the available material was adsorbed. On repeating the experiment described above, using a 7-Gm. column of resin, but using a solution of 0.01% w/v, the DBNS was readily adsorbed and the theoretical meq./Gm. dry weight resin was obtained. To check that this was not a pH effect, a solution of DBNS in the acid form (obtained by passing a solution of the sodium salt through Amberlite IRC 50) was used. This solution gave similar results to those obtained with the sodium salt.

Using Amberlite CG 400 type II, a very fine mesh resin (−200 +400 U. S. mesh) it was found that the maximum adsorption concentration can be considerably increased. A solution of DBNS of 0.17% w/v can be used in a batch-type process providing the adsorption temperature is raised to 40°. Using this method, a resin complex containing 335 mg. DBNS per dry 1.0 Gm. complex was obtained.

Fine mesh resin complex, 1 Gm., obtained by this batch process was suspended in 0.1 *N* hydrochloric acid and formed into a column. The resin was eluted with 500 cc. 0.1 *N* hydrochloric acid, and the concentration of DBNS in the eluate determined spectrophotometrically. The eluate contained 8.03 mg. DBNS. Another 500 cc. 0.1 *N* hydrochloric acid was passed through the same complex and was found to contain 7.42 mg. DBNS. By difference, the resin was known to contain 335 mg. DBNS. Similar results were obtained using other aqueous eluants such as 1% w/v sodium chloride, 1% w/v sodium hydroxide, 1% w/v sodium bicarbonate, 1 *N* hydrochloric acid, and 1 *N* sodium hydroxide. Fine mesh resin DBNS adsorbate, 1 Gm., was then eluted with 250 cc. 0.1 *N* hydrochloric acid in 95% v/v ethyl alcohol. This eluate was found to contain 393 mg. DBNS by spectrophotometric analysis. This was greater than the theoretical content, and the difference was thought to be due to a U. V. absorbing impurity which was extracted from the resin during elution with the alcoholic solvent.

The possibility of adsorbing DBNS on anion exchange resins from alcoholic solutions was determined. Tubes containing 250 mg. washed, cycled, rewashed, and dried resin were prepared and 25 cc. of DBNS solution of different concentrations was added to each tube. The tubes were rotated for one hour at room temperature and the percentage DBNS adsorbed determined spectrophotometrically by difference. This experiment was repeated using 20, 40, 60, and 95% v/v ethyl alcohol as solvent. The results are shown in Table II.

To determine whether the adsorption and elution effects described with DBNS were due to association colloid-type structure, or possibly some other steric phenomenon, adsorption and elution experiments using sodium dodecyl benzene sulfonate (DDBS) were carried out. Washed Amberlite CG 400 type II, 10 Gm., was stirred with 250 cc. of 0.17% w/v DDBS for one hour at room temperature. By difference, the dried resin complex was found to contain 39.1 mg. DDBS per 1.0 Gm. Elution of 1 Gm. of this complex with 500 cc. 0.1 *N* aqueous hydrochloric acid removed only 12.1 mg. DDBS from the resin.

DISCUSSION

Abrupt changes in the physical properties/concentration curves of association colloids have been reported by many authors. Ekwall (7, 8) has shown that measurements of equivalent conductance, solubilization properties, hydroxyl ion activity, and partial molal volume, when plotted against concentration, gave curves which showed a distinct break. Lamm and Högberg (9), Granath (10), Stigter, *et al.* (11), and Brudney and Saunders

Table II.—Percentage DBNS Adsorbed by Amberlite IRA 401 Using Various Alcoholic Solutions at Room Temperature

Percentage DBNS Soln.	Water	Percentage of DBNS Adsorbed			
		20% EtOH	40% EtOH	60% EtOH	95% EtOH
0.001	83.0	...	...	...	...
0.01	97.5	97.5	90.0	...	...
0.02	87.3	98.2	92.5	...	...
0.03	68.5	96.5	...	...	...
0.04	45.5	74.6	95.8	...	...
0.05	38.9	...	...	...	...
0.06	27.3	55.3	95.8	...	...
0.07	24.0	36.0	...	...	...
0.08	25.5	34.0	94.7	...	...
0.09	21.5	31.1	...	...	...
0.1	12.8	...	93.7	69.7	54.4
0.12	...	...	93.5	...	...
0.14	...	...	86.2	...	...
0.16	...	...	77.1	...	...
0.18	...	...	67.8	...	...
0.2	...	...	66.0	66.0	47.6
0.3	...	...	45.9	78.5	38.5
0.4	...	...	32.5	52.7	34.0
0.5	...	...	...	42.8	30.0
0.6	...	...	22.6	36.5	27.3
0.7	...	...	...	35.1	22.3
0.8	...	...	18.5	31.0	...
0.9	...	...	...	...	20.5
1.0	1.0	...	...	23.6	24.2

(12, 13), have all shown abrupt changes in the diffusion coefficient/concentration curves of various anionic and cationic, sulfonic, and carboxylic association colloids. It is, therefore, not too surprising that there should be an abrupt change in the adsorption/concentration curve of association colloids. This abrupt change is undoubtedly due to the formation of micelles, in these solutions at the critical micelle concentration (CMC). This is confirmed in the case of DBNS by the work of Yurzhenko and Kucher (14, 15, 16) who report on turbidity and light scattering measurements with solutions of sodium dibutylnaphthalene sulfonate. An abrupt change in the physical properties/concentration curve at 0.01% (0.0029 *M*) was found in all cases at 20°. This agrees very well with the results reported above.

The results described in this paper have been obtained with resins of different degrees of cross-linkage, but otherwise similar chemical constitution, indicating that the peculiar exchange properties are not a function of the resin, but of the association colloid. Further, the results obtained cannot be explained on the basis of ion exclusion, since solutions in water above the CMC cannot be adsorbed, but alcoholic solutions of equal ionic concentration can be adsorbed. The elution behavior of association colloids can, however, be readily explained on the basis of micelle formation. An association colloid, once adsorbed within a resin particle is undoubtedly adsorbed in the ionic form. As soon as an aqueous eluant such as 0.1 *N* hydrochloric acid is added to the complex, some of the ionic form is released from the adsorption sites by chloride ions, but as soon as sufficient material is released to attain the CMC, micelles are formed within the resin particles. The resin particles due to their mesh-like structure then act as a micellar sieve and very little association colloid is eluted. The actual amount of elution will depend on a number of factors such as the CMC, micellar volume, resin porosity and surface area, strength and nature of eluant, etc.

Adsorption from alcoholic solutions or elution with alcoholic solutions further confirms this explanation. The CMC is a function of alcohol concentration (17, 18) and is, in fact, inversely proportional at lower alcohol concentrations. This explains the necessity for alcoholic eluants in the analysis of detergents and shampoos (2–6). Newburger (2) found that alkyl sulfates when adsorbed onto weakly basic ion exchange resins could not be eluted by (1 + 9) ammonium hydroxide, dilute hydrochloric acid, ethyl alcohol, or an aqueous solution of ammonium hydroxide and ammonium carbonate. However, an alcoholic solution of either ammonium hydroxide or hydrochloric acid will strip the alkyl sulfate to some degree. Newburger conjectured that hydrophilic sulfate heads attach themselves to the basic resin, presenting a surface film of hydrophobic tails to the eluting solutions. The author explains, "The methanol makes it possible for the eluting ions to penetrate the hydrophobic layer and displace the alkyl sulfate from the resin." The results described in this paper indicate, however, that the explanation lies more correctly in the formation of micelles within the resin particles.

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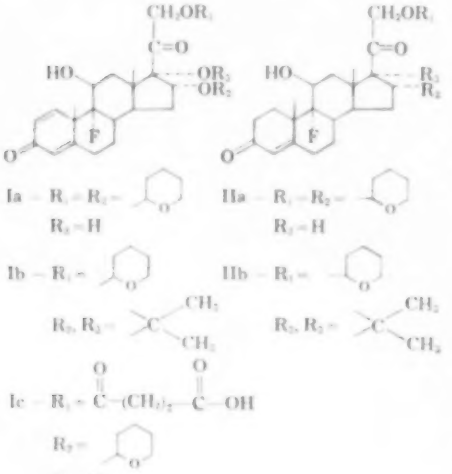
Some Tetrahydropyranyl Ether Derivatives of Steroid Hormones

By LEWIS J. LEESON and JOSEPH F. WEIDENHEIMER

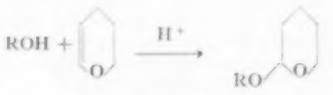
The 21-esters of adrenocortical steroid hormones are commonly-used materials in pharmaceutical formulations. Such derivatives as the 21-acetate, 21-*tert*-butylacetate, and 21-trimethylacetate are said to demonstrate a prolonged action and may be found in numerous parenteral preparations. The efficacy of these compounds is based on their decreased solubility in body fluids and the liberation of the more active parent steroid by enzymatic hydrolysis of the ester linkage (1–4).

The use of 21-ethers of adrenocorticoids is not nearly as common as the corresponding esters. Peterson and Gisvold (5) reported that tetrahydropyranyl ether derivatives of primary alcohols were the most stable compounds of a series prepared in their investigations with digitalis aglycones. Peterson (6) also determined the half life of 2-methoxytetrahydropyran to be from two and one-half to twelve hundred hours, depending on the acid concentration of the reacting media. It was, therefore, considered of interest to investigate the pharmacological properties of corresponding adrenocorticoid derivatives. For this reason a series of tetrahydropyranyl ethers of triamcinolone and some of its derivatives were prepared. These compounds are represented by general formulas I and II.



EXPERIMENTAL

The method of preparation of these steroid derivatives was based on the well-known reaction between an alcohol and 2,3-dihydropyran in the presence of an acid catalyst (7, 8). This reaction is often used as a means of blocking alcohol groups, and may be generally represented by the following equation



All compounds presented in this report were prepared in essentially the same manner, described as follows:

One gram of steroid was suspended in 50 ml. of freshly distilled 2,3-dihydropyran. A few drops of concentrated hydrochloric acid were added with mixing. The contents of the vessel became warm and, after a few hours, solution of the steroid was effected. Mixing was continued for an additional two to three hours, after which the solution was concentrated under vacuum at 35–40° to a viscous liquid. This material was poured into cold petroleum ether, with stirring, during which time a white precipitate formed. The solid was collected and recrystallized from such solvents as ethyl acetate, aqueous acetone, aqueous methanol, and chloroform-petroleum ether. Reaction yields were approximately 75–80%.

In some cases, before the solution was concentrated under vacuum, a few sodium hydroxide pellets were added to neutralize the acid catalyst. However, this step is not considered essential since, in either event, the results obtained were the same.

Ia. *9α - Fluoro - 11β,17α - dihydroxy - 16α,21 - di-(2 - tetrahydropyranyloxy) 1,4 - pregnadiene - 3,20 - dione.* Melting point 180–181° (uncorrected); E_{239} = 13,926; $[\alpha]_D^{25}$ = +27° (*c* = 0.815 Gm./100 ml. in CH_3OH; 2-dm. tube). Infrared maxima—8.80μ, 9.30μ, 9.65μ.

Anal.—Calcd for $C_{31}H_{40}FO_8$: C, 66.17; H, 7.70; F, 3.38. Found: C, 66.13; H, 7.81; F, 3.38.

Ib. *9α- Fluoro- 11β- hydroxy- 16α,17α - isopropylidenedioxy - 21 - (2 - tetrahydropyranyloxy) - 1,4 - pregnadiene-3,20-dione.* Melting point 185–189° (uncorrected); E_{238} = 14,000; $[\alpha]_D^{25}$ = + 61.5° (*c* = 1.102 Gm./100 ml. in $CHCl_3$; 1-dm. tube) Infrared maxima—8.85μ, 9.25μ, 9.62μ.

Anal.—Calcd. for $C_{29}H_{35}FO_7$: C, 67.16; H, 7.58; F, 3.66. Found: C, 66.91; H, 7.88; F, 3.54.

Ic. *9α - Fluoro - 11β,17α,21 - trihydroxy - 16α-(2 - tetrahydropyranyloxy) - 1,4 - pregnadiene -3,20 - dione-21-hemisuccinate.*—Melting point 160–163° (uncorrected); E_{238} = 16,200; $[\alpha]_D^{25}$ = +42.2° (*c* = 1.038 Gm./100 ml. in $CHCl_3$; 2-dm. tube). Infrared maxima—8.65μ, 8.80μ, 9.32μ, 9.60μ.

Anal.—Calcd. for $C_{30}H_{39}FO_{10}$: C, 62.27; H, 6.80; F, 3.28. Found: C, 61.89; H, 7.26; F, 3.54

IIa. *9α - Fluoro - 11β,17α - dihydroxy - 16α,21 - di-(2 - tetrahydropyranyloxy) - 4 - pregnene - 3,20 -dione.*—Melting point 154–156° (uncorrected); E_{239} = 16,394; $[\alpha]_D^{25}$ = +80° (*c* = 1.034 Gm./100 ml. in

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CH_3OH; 2-dm. tube). Infrared maxima—8.80μ, 9.30μ, 9.65μ.

Anal.—Calcd. for $C_{31}H_{41}FO_8$: C, 65.93; H, 8.03; F, 3.37. Found: C, 66.40; H, 8.36; F, 3.08.

IIb. *9α - Fluoro - 11β - hydroxy - 16,17 - isopropylidenedioxy - 21 - (2 - tetrahydropyranyloxy) - 4 - pregnene-3,20-dione.* Melting point 190–193° (uncorrected); E_{235} = 16,608; $[\alpha]_D^{25}$ = +130° (c = 1.068 Gm./100 ml. in $CHCl_3$; 2-dm. tube). Infrared maxima—8.85μ, 9.25μ, 9.62μ.

Anal.—Calcd. for $C_{29}H_{41}FO_7$: C, 66.90; H, 7.94; F, 3.65. Found: C, 67.26; H, 8.02; F, 3.50.

Compounds Ia and IIa were prepared from their corresponding free alcohols. The starting materials for Ib and IIb were the acetonide derivatives of the steroids (9). Triamcinolone 21-monohemisuccinate (10) was used to prepare Ic.

Preliminary biological testing[1] of Ib in rats demonstrated increased thymus involution with time over that of the parent acetonide.

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[1] The authors are indebted to Dr. I. Ringler and coworkers for preliminary animal screening data on compound Ib.

Effect of Gibberellic Acid on the Second Generation of *Datura stramonium* Linné

By NANCY L. RAGAN† and LEO A. SCIUCHETTI

Seeds were obtained from stramonium which had received treatments with gibberellic acid in 1957. Plants grown in 1958 from these seeds under greenhouse conditions were shorter than controls and demonstrated significantly reduced dry weights in their parts. The concentration of alkaloids was not materially altered. The gibberellin effect did not manifest itself in the second generation plants.

IN A PREVIOUS publication (1) it was demonstrated that, when *Datura stramonium* was treated with gibberellic acid (G. A.), significant increases in height, marked increases in dry weights of the shoots, and significant decreases in the concentration of the alkaloids in the shoots have been induced in the treated plants. Seeds were obtained from stramonium plants which had received, in 1957, (*a*) a two-spray treatment with 1,000 p. p. m. of G. A. (A series), (*b*) 100 p. p. m. of G. A. (B series), and (*c*) untreated plants (controls). Seventeen each of the the aforementioned groups were grown, in 1958, in soil in clay pots under greenhouse conditions. G. A. was not employed during the second generation studies. During a four-week growth period height measurements were taken twice weekly and the plants were observed for growth characteristics. On June 20, 1958, ten of the fifty-seven-day-old plants were picked at random from each group and harvested. Division of the plants into their morphological parts, pulverization, storage of the powdered material, and the alkaloid analyses were conducted in a manner described in a previous publication (1).

Generally, the controls were taller than either of the other two groups. The A and B series did not demonstrate the increased stem elongation and increased height that was noted in their parent plants. The average heights of each group at harvest time were about 21 cm. for controls, 17.5 cm. for the A series, and 16.3 cm. for the B series. Also, the latter two were more prone to insect attack than the controls. The weight increases, as shown previously in G. A.-treated stramonium (1), were not observed in the second generation. In general, significant reductions were noted in the weights of the plant parts (Table I). Rather, the trend was the reverse of that noted in the first generation, i.e., shorter instead of taller plants and reduced growth rather than increased growth.

The alkaloid analyses, employing pooled samples, demonstrated that the concentration of alkaloids in the organs of the A and B series varied only slightly from controls. The significant reductions noted in the shoots of the first generation (1) were not observed in the second generation. The total alkaloid content per plant and per plant organ (obtained by calculation) was generally significantly higher in the controls (Table II). The decreased content of alkaloids in series A and B, however, was due to less growth as indicated by dry weights rather than to an effect on the concentration of alkaloids in the plant organs.

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TABLE I.—WEIGHTS OF STRAMONIUM PLANT PARTS (AV./PLANT/GROUP)

Sample	Total Weight			Leaves and Tops			Stems			Roots		
	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %	Fresh, Gm.	Dry, Gm.	Control Dry Wt., %
Control	36.6	4.9	..	15.5	2.4	..	6.9	0.91	..	14.2	1.6	..
A series	25.8	3.6	73.5	12.3	2.0	83.3	4.8	0.67	73.6	8.7	0.96	60.0
B series	22.5	3.4	69.4	10.3	1.8	75.0	4.5	0.66	72.5	7.7	0.93	58.1

TABLE II.—TOTAL ALKALOID CONTENT[a] OF *Datura stramonium*[b]

Sample	Per Plant		Leaves and Tops		Stems		Roots	
	Alkaloids, mg.	Control, %	Alkaloids, mg.	Control, %	Alkaloids, mg.	Control, %	Alkaloids, mg.	Control, %
Control	12.2	..	8.6	..	2.6	..	1.0	..
A series	9.1	75	6.6	77	1.8	69	0.67	67
B series	7.7	63	5.3	62	1.8	69	0.58	58

[a] Alkaloid content for plant parts calculated from dry weights and alkaloid analyses data; total plant alkaloid = leaves and tops + stems + roots; based on av./plant/group. [b] Total alkaloids expressed as scopolamine.

DISCUSSION

The profound influence that the direct application of G. A. had on the growth and alkaloid formation in *Datura stramonium* was not observed in the untreated second generation. Characteristic gibberellin effects were lacking. Instead of taller plants and increased stem elongation, the second generation plants were shorter than controls. The concentration of alkaloids in the morphological parts was not altered, whereas decreased concentration of alkaloids has been the usual gibberellin response (1–5). The primary effect of the previous year's treatment on the second generation was an adverse effect on growth. This was also observed on the second-year growth of belladonna treated the previous year with G. A. (6).

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Communications

Radioactive Drugs in U. S. Pharmacopeia

Sir:

The United States Pharmacopeia, 16th rev., includes monographs on radiogold solution, sodium radiochromate solution, sodium radio-iodide solution, sodium radiophosphate solution, and others. The specifications, tests, and assay methods recommended for these preparations, when considered from a radiochemical and radiological point of view, are quite unsatisfactory.

According to the U. S. P. specifications, multiple-dose preparations of the radiopharmaceutical contain a suitable bacteriostatic agent. In an intense radiation field such as those afforded by multimillicurie sources, pronounced radiolysis or radiation decomposition of the added agent may occur. In commercial preparations, benzyl alcohol is commonly used as a bacteriostatic agent. The G value for benzyl alcohol is not reported but the value for alcohols in pure state is given as 4 to 12 (1); that is, approximately 4 to 12 molecules are permanently altered or decomposed for every 100 electron volts of beta-energy expended. In aqueous solutions, radiolysis of water yields highly reactive hydrogen, hydroxyl, and perhydroxyl radicals which augment the extent of decomposition (2). Thus, the presence of odoriferous compounds and the discoloration in these preparations as noted in the U. S. P. may be explained on this basis. Neither the toxicity to man of these radiation decomposition products nor their value as bacteriostatic agents have been determined; indeed, the extent of radiation decomposition of the added bacteriostatic agent in these preparations has not been evaluated.

The inclusion of benzyl alcohol in commercial preparations of the sodium radio-iodide solution can also cause anomalous results when the preparation is intended for diagnostic use. The hydrogen peroxide and the perhydroxyl radical formed from radiolysis of water can oxidize the iodide ion to yield hypoiodite ion which in turn iodinates the aromatic nucleus of benzyl alcohol. The metabolic fate of the iodo compound differs from that of the iodide *per se*. Our observation (3) shows a significant difference in iodine uptake in rats given tracer doses of carrier-free iodine^{131} intraperitoneally, with and without benzyl alcohol. The animals receiving iodine^{131} solutions without benzyl alcohol gave four hours postinjection an average thyroid uptake and urinary excretion of 11.2 and 22.3 per cent of the administered dose, respectively, while those receiving the same solutions with added benzyl alcohol (approximately 1 per cent) gave values of 7.2 and 63.7 per cent, respectively. This clearly supports the assumption that part of the iodine^{131} exists in an organic form in the presence of added benzyl alcohol.

Another inadequacy of the U. S. P. specification is in connection with the carrier-free radiophosphate preparations. Phosphate forms highly insoluble compounds with heavy metal ions. At carrier-free concentrations ($<10^{-9}M$), most heavy metal phosphates will remain in solution but tend to become adsorbed to container surfaces and to other particulate matter present in solution. Our experience in this laboratory (4) reveals that filtration of such a solution leads to a loss of from approximately 60 to 90 per cent of its activity, whereas other soluble phosphate solutions show no significant loss to the filter paper; the filtrable activity of the former, even though remaining soluble at the recommended pH 5.0–6.0 (5), showed a different tissue distribution in rats from that of a nonheavy metal phosphate. For example, at approximately twenty hours postintramuscular injection, 13.8 per cent of the heavy metal phosphate solution remained at the site of injection and 14 per cent accumulated in the liver, whereas 3 and 6 per cent, respectively, were observed for nonheavy metal phosphate solutions. Notwithstanding these findings, the preparation containing heavy metal ions meets all the requirements of the Pharmacopeia for radiophosphate, since the trace concentration of the metal ions present is beyond the sensitivity of the analytical method recommended.

There are also inconsistencies and inadequacies in the Pharmacopeia concerning the identification and assay of the radioactive preparations. For instance, the formula for calculating the activity of a sample solution is given as SD(A)/(B) for radiocyanocobalamin, radiochromate, and radiogold, but as 100 SD(A)/(B) for radio-iodide and radiophosphate. Under the "Identification" of radiophosphate, one is advised to determine the mass absorption coefficient making use of the procedure recommended for radio-iodide which is given in detail under "Absorption" in the "General Information and Procedures" (p. 833). Obviously, it is theoretically unsound to apply such a procedure without modification for phosphorus^{32}, which is a pure beta-emitter with a maximum range of 799 mg. cm.$^{-2}$ in water (6). The attenuation of beta-ray through matter depends upon many parameters such as the degree of forbidden transition, atomic number of the absorber, scattering, counting geometry, etc. (7–9), which influence the shape of the absorption curve and consequently make the mass absorption coefficient less satisfactory than the maximum range as a property for identification of beta-emitters.

The recommended method of assay for radioactivity of the various preparations in the U. S. P. calls for a comparison with a calibrated standard of the same isotope. Strict adherence to this recommendation may, in practice, present an insurmountable barrier. Either the supplemental use of a more permanent reference standard and/or the calibration by fixed counting geometry which is standardized with a known sample of identical isotopes whenever one becomes available, should be recommended. The types of counting instrument to be employed for assaying gamma-emitting and electron-capturing nuclides, such as gold^{198}, cobalt^{60}, and chromium^{51} are described in the Pharmacopeia as "suitable counting assembly"; this ambiguity only leads to confusion and is unsatisfactory.

The increasing use of radioisotopes for diagnosis and therapy in recent years stresses the need of a more strict standard and a clearer description of the test and assay methods in the Pharmacopeia for the radiopharmaceuticals. The use of bacteriostatic agents in radiopharmaceutical preparations should be discouraged; either heat sterilization or other means of sterilization should be employed. The purity of the carrier-free preparations, when in doubt, should be based on the analytical results obtained by neutron activation rather than by conventional chemical techniques because the latter lack the required sensitivity of detection. The counting techniques and methods of standardization of radioisotopes are diverse and complex. If they cannot be treated adequately under "General Information

and Procedures," then other authoritative references, such as the National Bureau of Standards Circular 594 and pertinent literature should be cited.

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Book Notices

Handbook of Microbiology. By Morris B. Jacobs and Maurice J. Gerstein. D. Van Nostrand Co., Inc., 120 Alexander St., Princeton, N. J., 1960. x + 322 pp. 16.5 × 24 cm. Price $8.50.

A compilation of 38 tables is designed to provide data for professional workers in microbiology as applied in different fields, including bacteriologists, biologists, serologists, immunologists, mycologists, sanitarians, chemists, public health workers, etc. Although the groupings are called tables, many are composed of series of descriptions and procedures.

Problems in Photosynthesis. By W. Bladergroen. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, Ill., 1960. xiii + 198 pp. 17 × 25 cm. Price $10.50.

The text in this book, after a basic introduction, is divided under the three main headings: Photochemical considerations, Energetics of photosynthesis, and the Chemistry of photosynthesis. References are given after each chapter, and author and subject indexes are appended.

Congres des Sciences Pharmaceutiques, Zurich 1959 (19th). Conférences et Communications. Selbstverlag des Schweizerischen Apotheker-Vereins, Zürich, 1960. xii + 667 pp. 17 × 24.5 cm. Paperbound.

The papers presented at the Scientific Section of the Federation Internationale Pharmaceutique are included in this compilation. The papers are printed in the language in which they were reported. Twenty-five of the 74 papers are in English including three on stability and stabilization of pharmaceutical preparations.

Lipide Chemistry. By Donald J. Hanahan. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1960. ix + 330 pp. 15 × 23 cm. Price $10.

The progress in the studies on lipide chemistry during the last ten years is reflected in the new and improved fractionation techniques, isolation procedures, and methods of assay discussed in this book. Difficulties in establishing with certainty the structure and purity of lipides isolated from natural sources are considered.

Catalysis. Vol. 7. Edited by Paul H. Emmett. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1960. vi + 378 pp. 15 × 23 cm. Price $13.50.

The series on catalysis is completed with volume 7 which covers: Cracking catalysts; Catalytic dehydration and hydration; Hydrocarbon oxidation; Oxidation of ammonia, ammonia and methane, carbon monoxide and sulfur dioxide; Mechanism of catalytic oxidation; and Miscellaneous catalytic oxidations.

Human Pituitary Hormones. Edited by G. E .W. Wolstenholme and Cecilia M. O'Connor. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1960. xii + 336 pp. 13.5 × 20.5 cm. Price $9.50.

The papers and discussions at the August 1959 Ciba Foundation Colloquia on Endocrinology are compiled in this, the thirteenth volume of the series. The symposium was devoted to considerations of the anterior pituitary hormones and their effects in man and animals.

The Chemistry of Thyroid Disease. By ROSALIND PITT-RIVERS and JAMSHED R. TATA. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, Ill., 1960. xii + 83 pp. 15 × 22.5 cm. Price $4.50.

Treated as a monograph in this book, the subject is presented under the following headings: Normal biochemical processes in the thyroid gland, Biochemical changes in hypothyroidism, Biochemical changes in hyperthyroidism, Immunological aspects of thyroid disorders, and Cancer of the thyroid. References and an index are appended.

Dietary Proteins in Health and Disease. By JAMES B. ALLISON and WILLIAM H. FITZPATRICK. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, Ill., 1960. viii + 86 pp. 15 × 22.5 cm. Price $4.50.

An interpretation of the biochemistry of food proteins, correlating complex structure with physiological function is given in this book. The test is divided under the headings: Basic concepts, Digestion, Nitrogen balance, Proteins and growth, Protein and maintenance, Calories and proteins, Nutritive value and problems in supplementation, and Plasma and liver proteins.

The Clinical Use of Aldosterone Antagonists. Edited by FREDERIC C. BARTTER. Charles C Thomas, 301-327 East Lawrence Ave., Springfield, Ill., 1960. xi + 211 pp. 15 × 22.5 cm. Price $5.

A symposium sponsored by G. D. Searle & Co., with twenty-three participants, is compiled in this book. Discussions on the fundamental mechanism of action and clinical results with aldosterone antagonists used in diagnosis and clinical therapy were included. An index is appended.

Controlled Clinical Trials. Organized under the direction of A. B. HILL. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, Ill., 1960. vii + 177 pp. 14 × 22.5 cm. Price $5.

The papers delivered at the Conference on Controlled Clinical Trials called by the Council for International Organizations of Medical Sciences in March 1959 make up this book. Methods of conducting trials and also the ethical problems involved were discussed. A list of selected references is appended, but no index is included.

Official Methods of Analysis of the Association of Official Agricultural Chemists. 9th ed. Edited by WILLIAM HORWITZ. Associaion of Official Agricultural Chemists, P. O. Box 540, Benjamin Franklin Station, Washington 4, D. C., 1960. xx + 832 pp. 17.5 × 25.5 cm. Price single copy $17.50 domestic, $18 foreign.

The ninth edition of "Official Methods of Analysis, AOAC," includes the procedures sponsored by the Association including those approved as "first action" or adopted as "official" at the 1959 meeting. As a source of analytical tests and assays for general testing laboratories, where drugs and dosage forms requiring procedures other than those in the U. S. P. and N. F. are frequently encountered, the book is an incomparable necessity.

The soundly established procedure of collaborative study of proposed methods and changes in older methods continues as the basis for acceptance. Purchasers of the 9th ed. can obtain, at cost, reprints of the changes in methods adopted at the annual meetings 1960–1963, inclusive.

The new double-column format and typographical changes have vastly improved the readability of the text while conserving space. The chapter on soils has been omitted but the scheme of elementary analysis has been retained by inclusion in the chapter on liming materials. Other editorial changes make for more convenient use of the book. This is an excellent revision of an important working and reference volume. The extensive subject index adds to its usefulness.

Cellular Aspects of Immunity. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1960. xii + 495 pp. Price $10.50.

The twenty reports and discussions and the group discussion in summary of the proceedings at the Ciba Foundation Symposium of June 3–5, 1959 are compiled in this volume. Material on the population dynamics of mesenchymal cells as they are modified and influenced by immunological processes was presented. The questions of how the immunological information that is lodged in an immunologically competent cell is conveyed to it, and how that information can be transmitted were discussed. Author and subject indexes are included.

The Nucleic Acids. Vol. 3. Edited by ERWIN CHARGAFF and J. N. DAVIDSON. Academic Press Inc., 111 Fifth Ave., New York 3, N. Y., 1960. xvi + 588 pp. 15 × 23 cm. Price $18.

Volume three in this progressive treatise includes twelve contributed chapters on: Deoxyribunucleic acids as macromolecules, Photochemistry of nucleic acids and their constituents, Chemical and enzymic synthesis of polynucleotides, Chemistry of the nucleic acids of microorganisms, Nucleic acids of the bacterial viruses, Ribonucleic acids of viruses, Biosynthesis of purine nucleotides, Biosynthesis of pyrimidine nucleotides, Relationship of nucleic acid and protein synthesis as revealed by studies in cell-free systems, Biosynthesis of proteins in intact bacterial cells, Agents which influence nucleic acid metabolism, and Effect of radiations on nucleic acid metabolism. The chapter headings and authors for Vols. 1 and 2 are given, and author and subject indexes for Vol. 3 are appended.

Quantitative Analysis. By RAY U. BRUMBLAY. Barnes & Nobles, Inc., 105 Fifth Ave., New York 3, N. Y., 1960. xvii + 235 pp. 13 × 21 cm. Paperbound. Price $1.50.

A concise presentation of material required for a one-semester course in quantitative analysis is given in this book. The text includes current procedures, but the 1955 revision of the Table of International Atomic Weights is appended. A good book for its limited purpose.

Internationales Biguanid-Symposium. Edited by F. BERTRAM and G. MICHAEL. Georg Thieme Verlag, Stuttgart, Germany, 1960. Available in the U. S. and Canada from Intercontinental Medical Book Co., 381 Fourth Ave., New York, 16, N. Y. vii + 167 pp. 15.5 × 23 cm. Paperbound. Price $4.

Reports and discussions on these with biguanides, including phenformin (DBI), are compiled from the 1960 symposium held at Aachen. All reports are in German, including two by U. S. groups, except one discussion by H. S. Sadow of New York which was not translated from the English.

Ultrafiltration. By L. AMBARD and S. TRAUTMANN. Charles C Thomas, 301–327 East Lawrence Ave., Springfield, Ill., 1960. x + 67 pp. 15.5 × 23 cm. Price $4.50.

Professor A. M. Monnier who translated this monograph from French to English notes in the preface that Ambard's book contains much more than the title indicates; it is a "reappraisal of fundamentals" introducing entirely new concepts. Theories and experimental results in ultrafiltration are related to biological phenomena. Monnier states that even if one is opposed to the views developed by Ambard, he will realize that current theories do not offer any straightforward account of the curious phenomena presented by ultrafiltration.

The Dispensatory of the United States of America. Vol. 2. New Drug Developments. Edited by ARTHUR OSOL and ROBERTSON PRATT. Medical Dept., J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1960. 240 pp. 17.5 × 26 cm. Price $9.

Volume 2 of U. S. D. 25 is mainly devoted to providing information concerning more than 200 new drugs and their many dosage forms that have been introduced since the publication of U. S. D. volume 1, in 1955. New information about certain drugs already described in volume 1, and general survey articles on antibiotics, antibiotics with antitumor activity, hypoglycemic sulfonylureas and biguanides, psychotherapeutic and psychotomimetic drugs, and saluretic agents are included. Chemical and trade names are given immediately under the generic title of each monograph. The concise thoroughness of U. S. D. is maintained.

Essentials of Pharmacology. 4th ed. By FRANCES K. OLDHAM, F. E. KELSEY, and E. M. K. GEILING. Medical Dept., J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1960. xiv + 418 pp. 15 × 23 cm. Price $7.75.

A concise, yet broad survey of pharmacology is presented for the undergraduate student. Added in this edition is a chapter on hallucinogens and tranquilizers, and a section on monoamine oxidase inhibitors under CNS stimulants. Although the existence of U. S. P. XVI and N. F. XI are recognized in chapter 3, page 16, the preparations listed at the end of each chapter were compiled from U. S. P. XV and N. F. X in addition to the B. P. and N. N. D.

American Pharmacy. 5th ed. Edited by JOSEPH B. SPROWLS. Medical Dept., J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1960. xxiii + 493 pp. 18 × 25.5 cm. Price $10.75.

The fifth edition of this textbook of pharmaceutical principles, processes, and preparations has incorporated the changes necessary to bring it into agreement with U. S. P. XVI, N. F. XI, and the N. N. D. 1960.

Biology Code of the Chemical-Biological Coordination Center. Vol. 1, Code. Vol. 2, Key to the Code. Edited by PHILIP G. SEITNER. National Academy of Sciences, National Research Council, Washington 25, D. C., 1960. Vol. 1, viii + 222 pp. Vol. 2, iv + 210 pp. 21.5 × 27.5 cm. Paperbound. Price $12 a set or $7 each volume. Also available in cloth binding.

Developed by the Chemical Biological Coordination Center, the code was used for coding (indexing) the collected information for IBM punched card storage. The data collected from 1945 until the Center was terminated in 1957 covers the chemical-biological fields (pharmaceutical and clinical, plant growth regulation, insecticide, etc.) Volume 1 contains the series of classification schemes, code items, and code symbols. Volume 2, "Key to the Biology Code," describes each of the lists and gives explanations and instructions for the use of the code symbols.

Chemobiodynamics and Drug Design. By F. W. SCHUELER. The Blakiston Division, McGraw-Hill Book Co., Inc., 330 West 42nd St., New York 36, N. Y., 1960. xiv + 638 pp. 15 × 23 cm. Price $19.50.

The principles of chemical control in biology are applied by the author to the problems of drug design. Part 1, entitled "Chemobiodynamics," emphasizes the structural and functional relationship of chemistry to biology and its antecedents. Part 2 emphasizes the nature of drug design as an activity preceding drug synthesis. The relationship between chemical constitution and pharmacological activity is developed throughout the text, and the fundamentals of drug action is well presented. References are given in the text and an index is appended.

Lipide Metabolism. Edited by KONRAD BLOCH. John Wiley & Sons, Inc. 440 Fourth Ave., New York 16, N. Y., 1960. xiii + 411 pp. 15 × 23 cm. Price $10.50.

Planned as a companion to Hanahan's "Lipide Chemistry" this book is a series of contributions dealing with the transformations of lipides by living systems and by isolated enzymes. The text is divided under the headings: Enzymatic mechanisms of fatty acid oxidation and synthesis, Metabolism of unsaturated fatty acids, Metabolism and function of phosphatides, Metabolism of glycerides, Lipolytic enzymes, Hormonal regulation of fatty acid metabolism, Formation and metabolism of bile acids, and Chemistry and metabolism of bacterial lipides. References are given, alphabetically, at the end of each chapter, and an index is appended.

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